REMARKS

Applicants cancel, without prejudice, claims 5-6, 10-12, 14-18, 21-30 and 33-47. Applicants reserve the right to prosecute claims with identical or similar scope in one or more future continuation or divisional applications.

Claim 1 has been amended to recite three CDRs from the variable light region of the 1G4 antibody and three CDRs from the variable heavy region of the 1G4 antibody. The claim amendments are fully supported by the specification.

Claims 13, 19 and 31 have been amended to improve their form and to more particularly point out what applicants intend to pursue in this application and to make the claims dependent on claim 1. The claim amendments are fully supported by the specification.

New claims 48-52 have been added.

Support for the amendments made herein and new claims can be found in the original claims and the specification at, e.g.: page 5, line 25 to page 6, line 2; page 6, line 17 to page 7, line 3; and page 12, lines 26 to 28. Support for the claims can also be found in Figs. 4a and 4b of the application as filed, which figures set forth, *inter alia*, the amino acid sequences for the light chain variable region and heavy chain variable region of the monoclonal anti-DC-SIGN antibody 1G4. Note that the elected sequence depicted in SEQ ID NO:45 is the amino acid sequence of the light chain CDR3 of 1G4. *See* Fig. 4a. At the time the application was filed, it was well within the purview of the ordinarily skilled artisan to determine the position of the instantly claimed CDRs of a light chain variable region and/or a heavy chain variable region. For example, by comparing the 1G4 variable region amino acid sequences with the variable region amino acid sequences of numerous other murine antibodies, the skilled artisan could have easily and readily determined the "Kabat"-defined CDRs. *See*, e.g., Kabat *et al.* (1991) "Sequences of Proteins of Immunological Interest." NIH Publication No. 91-3242, U.S. Department of Health and Human Services, Bethesda, MD. Accordingly, no new matter has been added.

Applicants' cancellation of and amendments to the claims are not in acquiescence to any of the Examiner's rejections. Applicants reserve the right to further prosecute the same or similar claims in the instant application or in one or more subsequent patent applications claiming priority to the instant application.

Upon entry of the amendments, claims 1-4, 7-9, 13, 19-20, 31-32 and 48-52 will be pending. No new matter has been introduced. Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the outstanding Office Action.

DETAILED ACTION

Restriction Requirement

Applicants note with appreciation that the response to the Restriction Requirement submitted on June 8, 2009 has been received by the office and that the specific method species election requirement has been withdrawn.

Claim Rejections – 35 U.S.C. 112, First Paragraph, Enablement

Claims 1-9, 12-13, 19-20, 31-32 and 35 are rejected under 35 U.S.C. 112, first paragraph, for allegedly failing to enable one of skill in the art to practice the claimed invention. Specifically, the Examiner states that there is no evidence of record that an antibody defined by a single CDR could bind DC-SIGN nor function as claimed in the dependent claims 5-6, 19 or 35 without undue experimentation. The Examiner also states that claim 19 requires that the claimed antibodies "can effectively block the binding, ***infection, or transmission*** of numerous viruses, bacteria, and parasites[,]" which the Examiner argues is not enabled. The Examiner also asserts that claim 19 is non-enabled because the specification allegedly does not provide, e.g., "[d]ata regarding the treatment or prevention of any disease or condition[.]"

Applicants disagree. Nonetheless, solely to expedite prosecution, applicants have amended claims 1, 13, 19 and 31 and canceled claims 5-6, 12 and 35. The remaining claims, as amended, are now directed to antibodies that are defined by six (6) CDRs – three from the variable light region of the 1G4 antibody and three from the variable heavy region of the 1G4 antibody. Therefore, this aspect of the rejection is rendered moot.

Applicants point out that claim 19 is not drawn to a method for ***treating or preventing any disease or condition*** nor is it specifically drawn to antibodies that block the binding, infection, or transmission of ***any*** virus, bacteria, or parasite. Rather, claim 19 is drawn to antibodies that are capable of effectively blocking the binding, infection, or transmission of a select number of microbial pathogens to cells that express DC-SIGN.

Methods for determining whether the claimed antibodies block the binding of one of the selected microbes to a cell expressing DC-SIGN were well known in the art at the priority date of the application and specifically described in the specification. At Example 8, the specification describes a FACS-based method for selecting anti-DC-SIGN antibodies capable of blocking the binding of microorganisms to DC-SIGN-expressing cells. The specification further cites to Geijtenbeek *et al.* (1999) *Blood* 94:754 as elaborating on the method. Thus, it was well within the skill set of the artisan to identify the instantly claimed antibodies that block the binding of selected pathogenic microbes to a DC-SIGN-expressing cell.

Similarly, methods for determining whether the claimed antibodies block the infection of one of the selected microbes to a cell expressing DC-SIGN, or the transmission of the microbe by the cell to another cell, were also well known in the art and described in the specification. For example, the specification provides methods for inhibiting the transmission of a virus from one cell to another and methods for assessing whether an antibody can block infection of a cell by a bacterium in Examples 10 and 11, respectively. The specification cites to numerous scientific references describing such methods. *See* specification at pages 25 and 26. Thus, it was also well within the skill set of the artisan to identify the instantly claimed antibodies that block the infection

of DC-SIGN-expressing cells by the selected microbes recited in the claims or that block the transmission of the microbes by the cells.

While the Examiner concedes that HIV binds to DC-SIGN expressing cells and facilitates the entry of the virus into DC-SIGN expressing cells, the Examiner argues that "there is no nexus between said HIV binding and the binding, infection, or transmission of any of the other pathogens of the claim." Office Action at page 4. Applicants disagree, believing that the state of the art at the time the application was filed taught against the Examiner's conclusion. First, it was well known in the art that DC-SIGN binds to the high mannose-containing glycoproteins present on the surface of pathogenic microbes such as HIV. *See*, e.g., Geijtenbeek *et al.* (2000) Cell 100(5):587-597 (the authors stating that "[w]e describe the properties of ... DC-SIGN, that is highly expressed on [dendritic cells] present in mucosal tissues and binds to the HIV-1 envelope glycoprotein gp120."). Accordingly, it is reasonable to conclude, as the inventors did, that, e.g., other microbes that express mannose-containing glycoproteins may also bind to DC-SIGN in a similar way. In fact, Lozach *et al.* (2004) *J Biol Chem* 279(31):32035-32045 describe that DC-SIGN binds to the mannose glycans present on the surface of Hepatitis C virus (HCV). DC-SIGN was also known to bind to the surface proteins of Ebola [Alvarez *et al.* (2002) *J Virol* 76:6841-6844] and cytomegalovirus (CMV) [Halary *et al.* (2002) *Immunity* 17:653-664]. Therefore, in stark contrast to the Examiner's assertion, the art makes clear that there was a nexus between HIV binding to a DC-SIGN expressing cell and the binding of the other viruses recited in the claims to the cells.

Moreover, the ability of the selected microbes to infect DC-SIGN-expressing cells (e.g., dendritic cells) was also known in the art at the priority date of the application. For example, the capability of, e.g., Ebola virus, cytomegalovirus, *Leishmania pifanoi*, *Mycobacterium tuberculosis*, as well as HIV to infect DC-SIGN-expressing dendritic cells was discussed in, e.g., Tassaneetrithep *et al.* (2003) *J Exp Med* 197(7):823-829. *See also* Lozach *et al.* (2004), *supra*; Alvarez *et al.* (2002), *supra*; and Halary *et al.* (2002), *supra*. In addition, it was well known in the art that anti-DC-SIGN antibodies could block the transmission of certain microbes by an infected cell to another cell. *See*, e.g., Cormier *et al.* (2004) *Proc Natl Acad Sci USA* 101(39):14067-14072.

In view of the foregoing, it is clear that the skilled artisan reading the instant specification at the time the application was filed would have readily and easily understood: (a) the relationship between the selected microbes recited in claim 19 and their ability to bind to and infect DC-SIGN-expressing cells as well as to be transmitted from the cell to another; and (b) how to make, use, and identify an anti-DC-SIGN antibody that possesses the ability to block one or more of the processes in (a). Accordingly, reconsideration and withdrawal of this rejection are requested.

Claim Rejections – 35 U.S.C. 112, First Paragraph, Written Description

Claims 1-9, 12-13, 19-20, 31-32 and 35 are rejected under 35 U.S.C. 112, first paragraph, for allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. Specifically, the Examiner alleges that there is insufficient written description to show that applicants were in possession of an isolated antibody that binds DC-SIGN described only by the CDR of SEQ ID NO:45.

Applicants disagree. Nonetheless, solely to expedite prosecution, applicants have amended claims 1, 13, 19 and 31 and canceled claims 5-6, 12 and 35. The remaining claims, as amended, are now directed to antibodies that are defined by six (6) CDRs – three from the variable region of the 1G4 antibody and three from the variable heavy region of the 1G4 antibody. Therefore, this rejection is rendered moot.

CONCLUSION

In view of the above amendments, Applicants believe the pending application is in condition for allowance. The Examiner is invited to telephone the undersigned to discuss any issue pertaining to this response. Applicants request favorable consideration of the application and early allowance of the pending claims.

Applicants believe no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 18-1945, under Order No. ALEX-P01-112 from which the undersigned is authorized to draw.

Dated: December 29, 2009

Respectfully submitted,

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DC-SIGN, a Dendritic Cell–Specific HIV-1-Binding Protein that Enhances *trans*-Infection of T Cells

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Summary

Dendritic cells (DC) capture microorganisms that enter peripheral mucosal tissues and then migrate to secondary lymphoid organs, where they present these in antigenic form to resting T cells and thus initiate adaptive immune responses. Here, we describe the properties of a DC-specific C-type lectin, DC-SIGN, that is highly expressed on DC present in mucosal tissues and binds to the HIV-1 envelope glycoprotein gp120. DC-SIGN does not function as a receptor for viral entry into DC but instead promotes efficient infection in *trans* of cells that express CD4 and chemokine receptors. We propose that DC-SIGN efficiently captures HIV-1 in the periphery and facilitates its transport to secondary lymphoid organs rich in T cells, to enhance infection in *trans* of these target cells.

Introduction

Transmission of human immunodeficiency virus type 1 (HIV-1) infection in humans requires the dissemination of virus from sites of infection at mucosal surfaces to T cell zones in secondary lymphoid organs, where extensive viral replication occurs in $CD4^+$ T-helper cells (Fauci, 1996). These cells express both CD4 and the chemokine receptor CCR5, which together form the receptor complex required for entry by the R5 viral isolates that are prevalent early after infection (Dragic et al., 1996; Lu et al., 1997; Littman, 1998). Viruses with tropism for other chemokine receptors, particularly CXCR4, are rarely transmitted and generally appear only late in infection.

The mechanism of early viral dissemination remains vague, but based on anatomical distribution of different hematopoietic lineage cells and on in vitro infectivity studies it has been inferred that immature dendritic cells (DC) residing in the skin and at mucosal surfaces are the first cells targeted by HIV-1. DC are the most potent antigen-presenting cells in vivo (Valitutti et al., 1995; Banchereau and Steinman, 1998). Immature DC in peripheral tissues capture antigens efficiently and have the unique capacity to subsequently migrate to the T cell areas of secondary lymphoid organs. As the cells travel, they mature and alter their expression profile of cell surface molecules, including chemokine receptors, lose their ability to take up antigen, and acquire competence to attract and activate resting T cells in the lymph nodes (Adema et al., 1997; Banchereau and Steinman, 1998). HIV-1 is thought to subvert the trafficking capacity of DC to gain access to the $CD4^+$ T cell compartment in the lymphoid tissues (Grouard and Clark, 1997; Rowland-Jones, 1999; Steinman and Inaba, 1999).

Immature DC express CD4 and CCR5, albeit at levels that are considerably lower than on T cells (Granelli-Piperno et al., 1996; Rubbert et al., 1998), and they have been reported to be infectable with R5 strains of HIV-1. In contrast, immature DC do not express CXCR4 and are resistant to infection with X4 isolates of HIV-1 (Weissman et al., 1995; Blauvelt et al., 1997; Granelli-Piperno et al., 1998). Entry of HIV-1 into immature DC has also been reported to proceed through a CD4-independent mechanism (Blauvelt et al., 1997), suggesting that receptors other than CD4 could be involved. There have been conflicting reports regarding the significance of HIV-1 replication within DC (Cameron et al., 1994; Ayehunie et al., 1997; Canque et al., 1999). Although replication can be observed in some circumstances, it has also been reported that, in immature DC, replication is incomplete and that only early HIV-1 genes are transcribed.

It has been proposed that virus-infected immature DC migrate to the draining lymph nodes where they initiate both a primary antiviral immune response and a vigorous productive infection of T cells, allowing systemic distribution of HIV-1 (Cameron et al., 1992; Weissman et al., 1995; Granelli-Piperno et al., 1999). However, in a nonhuman primate model of mucosal infection with the simian immunodeficiency virus, it has been difficult to demonstrate productive infection of DC despite rapid dissemination of virus (Stahl-Hennig et al., 1999). Other efforts to model primary HIV-1 infection in vitro by exposing DC derived from skin or blood to HIV-1 have indicated that these cells are poorly infected. Nevertheless, only DC and not other leukocytes, including monocytes, macrophages, B cells, and T cells, were able to induce high levels of infection upon coculture with mitogen-activated $CD4^+$ T cells after being pulsed with HIV-1 (Cameron et al., 1992, 1992b, 1996; Weissman et al., 1995; Blauvelt et al., 1997; Granelli-Piperno et al., 1999). In an early study, Cameron et al. (1992) proposed that DC have a unique ability to "catalyze" infection of T cells with HIV but do not become infected themselves.

The mechanism by which DC capture HIV-1 and promote infection of $CD4^+$ T cells has not been elucidated,

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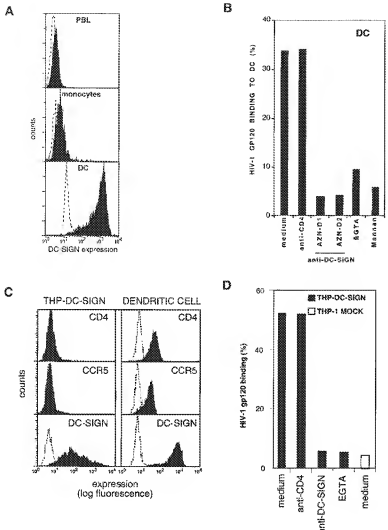


Figure 1. DC-SIGN Is a DC-Specific Receptor for HIV-1 gp120

(A) DC-SIGN is expressed specifically by DC. Immature DC, cultured from monocytes in the presence of GM-CSF and IL-4, express high levels of DC-SIGN, whereas resting peripheral blood lymphocytes and monocytes do not express DC-SIGN. Expression of DC-SIGN (AZN-D1) was determined by FACScan analysis. One representative experiment out of three is shown.

(B) DC-SIGN, but not CD4, mediates binding of HIV-1 gp120 to DC. DC were allowed to bind HIV-1 gp120-coated fluorescent beads. Adhesion was blocked by anti-DC-SIGN antibodies (20 μg/ml), mannan (20 μg/ml), and EGTA (5 mM), and not by neutralizing anti-CD4 antibodies (20 μg/ml). One representative experiment out of three is shown.

(C) Immature DC express low levels of CD4 (RPA-T4) and CCR5 (2D7/CCR5) and high levels of DC-SIGN (AZN-D1). THP-1 cells stably transfected with DC-SIGN (THP-DC-SIGN) express high levels of DC-SIGN (AZN-D1) while CD4 and CCR5 are not expressed (filled histograms). Antibodies against CD4 and DC-SIGN were isotype matched, and the appropriate isotype controls are represented by dotted lines.

(D) DC-SIGN transfectants (THP-DC-SIGN) bind HIV-1 gp120. THP-DC-SIGN and mock transfectants were allowed to bind HIV-1gp120-coated fluorescent beads. Adhesion was blocked by anti-DC-SIGN antibodies (20 μg/ml) and EGTA (5mM) and not by neutralizing anti-CD4 (RPA-T4) antibodies (20 μg/ml). One representative experiment out of three is shown.

and it has been unclear whether there is specificity in the interaction of DC with virus. In the accompanying paper, we describe the identification of a DC-specific C-type lectin, designated DC-SIGN, that binds with high affinity to ICAM-3 present on resting T cells (Geijtenbeek et al., 2000 [this issue of *Cell*]). Nucleotide sequence analysis of the cDNA indicated that this molecule is identical to a previously described HIV-1 gp120-binding C-type lectin (Curtis et al., 1992) isolated from a placental cDNA library. Here, we demonstrate that this HIV-1-binding protein, which is highly expressed on DC present at mucosal sites, specifically captures HIV-1 and promotes infection in *trans* of target cells that express CD4 and appropriate chemokine receptors. Our findings suggest that, during transmission of HIV-1, the virus initially binds to mucosal DC through DC-SIGN, allowing subsequent transport to secondary lymphoid organs and highly efficient infection of $CD4^+$ T cells by a novel *trans* infection mechanism.

Results

DC-SIGN Is a DC-Specific HIV-1-Binding Protein

DC-SIGN was recently identified as a DC-specific ICAM-3 adhesion receptor that mediates DC-T cell interactions (Geijtenbeek et al., 2000). Flow cytometric analysis of an extensive panel of hematopoietic cells with anti-DC-SIGN antibodies demonstrated that DC-SIGN is preferentially expressed on in vitro cultured DC but not on other leukocytes, such as monocytes and peripheral blood lymphocytes (PBL) (Figure 1A). Identification of DC-SIGN by peptide amino acid sequencing of the 44 kDa immunoprecipitated protein revealed it to be 100% identical in its amino acid sequence to the HIV-1 envelope glycoprotein gp120-binding C-type lectin previously isolated from a placental cDNA library (Curtis et al., 1992). To determine whether this molecule has a role in binding of HIV to DC, we used a flow cytometric adhesion assay (Geijtenbeek et al., 1999) to examine the ability of HIV-1 gp120-coated fluorescent beads to bind to immature DC (Figure 1B). The gp120-coated beads bound efficiently to the DC, and the binding was completely blocked by the anti-DC-SIGN antibodies AZN-D1 and AZN-D2. In contrast, neutralizing anti-CD4 antibodies had no effect on gp120 binding to DC. This result indicates that, although the primary HIV-1 receptor CD4 is expressed on DC (Figure 1C), HIV-1 gp120 preferentially binds to DC-SIGN. Similarly, the monocytic cell line THP-1, which lacks expression of both CD4 and CCR5, bound the gp120-coated beads after

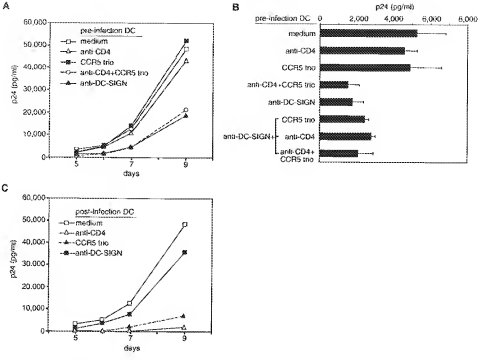


Figure 2. DC-SIGN Mediates HIV-1 Infection in a DC-T Cell Coculture

(A) Antibodies against DC-SIGN inhibit HIV-1 infection as measured in a DC-T cell coculture. DC (50×10^3) were preincubated for 20 min at room temperature with blocking mAb against CD4 (RPA-T4) or DC-SIGN (AZN-D1 and AZN-D2) (20 μg/ml) or with a combination of CCR5-specific chemokines (CCR5 trio: RANTES, MIP-1α, and MIP1β; 500 ng/ml). Preincubated immature DC were pulsed for 2 hr with HIV-1 (M-tropic $HIV\text{-}1_{BAL}$ strain), and unbound virus particles and mAb were washed away. Subsequently, DC were cocultured with activated PBMC (50×10^3) for 9 days. Coculture supernatants were collected, and p24 antigen levels were measured by ELISA. One representative experiment out of two is shown.

(B) Inhibition of HIV-1 infection in a DC-T cell coculture by blocking DC-SIGN, CD4, and CCR5. HIV-1 replication in the DC-T cell coculture at day 5 of the experiment is described in Figure 5A. The results of day 5 are representative for days 6, 7, and 9 of DC-T cell coculture. DC were also preincubated with mAb against DC-SIGN together with anti-CD4 and CCR5-specific chemokines. p24 values represent mean ± SD of triplicate cultures. One representative experiment out of two is shown.

(C) DC-SIGN interactions with ICAM-3 are not involved in the transmission of DC-bound-HIV-1 to T cells. DC (50×10^3) were pulsed for 2 hr with HIV-1 (M-tropic $HIV\text{-}1_{BAL}$ strain), washed, and cocultured with activated PBMC (50×10^3) for 9 days in the presence of the CCR5-specific chemokines (CCR5 trio: RANTES, MIP-1α, and MIP1β; 500 ng/ml) or mAb against CD4 (RPA-T4) and DC-SIGN (AZN-D1 and AZN-D2) (20 μg/ml). Antibodies were added post-HIV-1 infection of DC, prior to the addition of PBMC. One representative experiment out of two is shown.

it was transfected with a DC-SIGN expression vector (Figure 1C). HIV-1 gp120 binding to this cell line, THP-DC-SIGN, was also blocked by anti-DC-SIGN antibodies, but not by anti-CD4 (Figure 1D). Binding of HIV-1 gp120 to DC-SIGN expressed on DC or THP-DC-SIGN was also inhibited by the carbohydrate mannan or EGTA, consistent with previous findings (Curtis et al., 1992) and with the observation that DC-SIGN is homologous to other members of the Ca^{2+}-binding mannose-type lectins (Weis et al., 1998). Together, these results demonstrate that DC-SIGN is a specific dendritic cell surface receptor for the HIV-1 envelope glycoprotein.

DC-SIGN Is Required for Efficient HIV-1 Infection in DC-T Cell Cocultures

Because DC-SIGN is exclusively expressed on DC and has a high affinity for HIV-1 gp120, we reasoned that it might play an important role in HIV-1 infection of DC or of T cells that make contact with DC. Immature DC, which express low levels of CD4 as well as CCR5 and abundant DC-SIGN (Figure 1C), were pulsed with the R5 isolate $HIV\text{-}1_{BAL}$ for 2 hr, washed, and cultured in the presence of activated T cells (Figures 2A and 2B). To determine the contribution of each of these receptors in this assay system, we examined the effects of antibodies against CD4 and DC-SIGN and of a combination of three CCR5-specific chemokines (RANTES, MIP-1α, and MIP-1β). Preincubation of the immature DC with antibodies against DC-SIGN prior to infection resulted in significant inhibition of HIV-1 replication (Figure 2A). Neither anti-CD4 nor the CCR5-specific chemokines inhibited on their own, although a combination of these did block infection of DC (Figure 2A), which is probably due to efficient inhibition of the T cell infection by (un)bound anti-CD4/chemokines. Activated T cells challenged with the same viral load exhibited a weaker infection than those cultured with virus-pulsed DC (data not shown).

Since DC-SIGN binds to ICAM-3 on T cells, it is possible that antibodies against DC-SIGN could interfere with the DC-T cell interaction and thereby prevent HIV-1 transmission. To examine this possibility, antibodies against DC-SIGN were added after exposure of DC to HIV-1 but prior to the addition of activated T cells. In

this setting, only CCR5-specific chemokines and anti-CD4 antibody strongly inhibited HIV-1 infection of activated T cells, while antibodies against DC-SIGN had no effect (Figure 2C). These results thus suggest that DC-SIGN has an important function in propagation of HIV-1 in DC-T cell cocultures and that this function is related to the ability of DC-SIGN to bind to gp120 and not to its interaction with ICAM-3.

DC-SIGN Does Not Mediate HIV-1 Entry

To investigate whether DC-SIGN acts as a receptor that permits HIV-1 entry, similar to CD4 plus CCR5, we studied HIV-1 entry into 293T cells that expressed either DC-SIGN (293T-DC-SIGN) or CD4 and CCR5 (293T-CD4-CCR5). Cells were pulsed overnight with HIV_{BA-L} and washed the next day, and p24 levels were determined. There was no detectable p24 protein in the culture supernatants harvested from 293-DC-SIGN cells several days after the HIV-1 pulse, whereas the 293T-CD4-CCR5 cells were readily infected (Figure 3A).

To examine the possibility that DC-SIGN may work in conjunction with either CD4 or CCR5 to permit viral entry, we extended the studies by using HIV-1 pseudotyped with the envelope glycoprotein of the R5 isolate $HIV\text{-}1_{ADA}$. We employed a replication-defective HIV-1 genome that encoded a luciferase reporter gene, which allows a quantitative measure of the levels of single-round infection (Figure 3B) (Deng et al., 1996). Transiently transfected 293T cells expressing either CCR5 (293T-CCR5), CD4 (293T-CD4), or both (293T-CD4-CCR5), in the presence or absence of DC-SIGN, were infected with the reporter virus, and luciferase levels were determined after 2 days. As observed with replicating virus, HIV-1 entry was not detected in 293T cells that expressed only DC-SIGN (Figure 3B). No infection was observed if DC-SIGN was expressed with either CD4 or CCR5, indicating that DC-SIGN does not form a complex with these molecules to permit viral entry. In contrast, high luciferase activity was obtained after infection of 293T cells expressing both CD4 and CCR5, and expression of DC-SIGN did not contribute further to viral entry into these cells (Figure 3B). Therefore, DC-SIGN cannot substitute for CD4 or CCR5 in the process of HIV-1 entry.

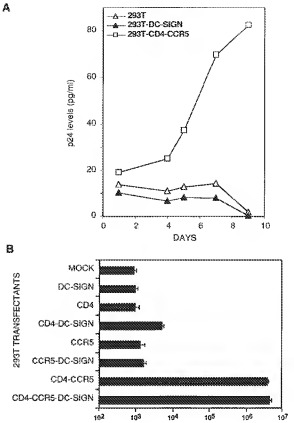


Figure 3. DC-SIGN Expressed on Target Cells Does Not Mediate HIV-1 Entry

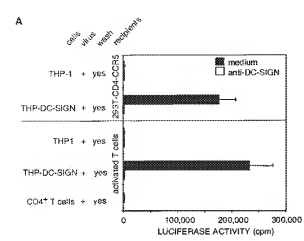
(A) 293T cells were transfected with DC-SIGN or CD4 and CCR5 and pulsed for 2 hr with HIV-1 (CCR5-tropic $HIV\text{-}1_{BA-L}$ strain). Subsequently, cells were cultured for 9 days. Supernatants were collected, and p24 antigen levels were measured by ELISA. One representative experiment of two is shown.

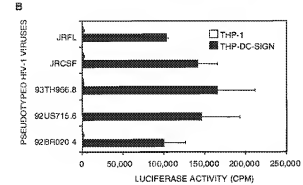
(B) 293T cells and 293T cells stably expressing either CD4, CCR5, or CD4 and CCR5 were transiently transfected with DC-SIGN and subsequently infected with pseudotyped CCR5-tropic $HIV\text{-}1_{ADA}$ virus in the presence of polybrene (20 μg/ml). Luciferase activity was evaluated after 2 days. One representative experiment out of three is shown.

DC-SIGN Captures HIV-1 and Facilitates Infection of HIV-1 Permissive Cells In *trans*

Because DC-SIGN did not appear to mediate virus entry into target cells, we hypothesized that in a DC-T cell coculture (Figure 2) DC-SIGN might facilitate both capture of HIV-1 on DC, independent from CD4 and CCR5, and subsequent transmission of HIV-1 to the CD4/CCR5-positive T cells. To test this, THP-DC-SIGN transfectants, which do not express CD4 or CCR5 (Figure 1C) and which cannot be infected by HIV-1 (data not shown), were pulsed with single-round HIV-luciferase virus pseudotyped with the $HIV\text{-}1_{ADA}$ envelope glycoprotein. After washing to remove unbound virus, the cells were cocultured with CD4/CCR5-expressing 293T cells, which are permissive for HIV-1 infection, or activated T lymphocytes. THP-DC-SIGN cells were able to capture the pseudotyped virus and transmit it to the target cells that expressed the receptors required for viral entry (Figure 4A). HIV-1 capture was completely DC-SIGN dependent, as antibodies against DC-SIGN inhibited HIV-1 infection (Figure 4A), and DC-SIGN-negative parental THP-1 cells were unable to capture and transmit HIV-1 (Figures 4A and 4B). Similar to our previous findings, the DC-SIGN-mediated infection of the target cells was not due to DC-SIGN binding to ICAM-3, since 293T cells are ICAM-3 negative. These findings indicate that DC-SIGN expressed at the surface of heterologous cells can capture HIV-1 in a form that retains its capacity to subsequently infect HIV-1-permissive cells. The ability of DC-SIGN to capture and transmit HIV-1 was also observed with HIV-luciferase viruses pseudotyped with envelope glycoproteins from an additional five R5 isolates, including three primary isolates (Figure 4B), and from the X4 isolate HXB2 (data not shown).

Analysis of luciferase activity in both adherent (293T-CD4-CCR5) and nonadherent (THP-DC-SIGN) cell frac-





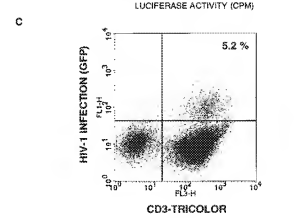


Figure 4. DC-SIGN Captures HIV-1 that Retains Infectivity for $CD4^+$ T Cells

(A) DC-SIGN captures HIV-1 and facilitates infection of HIV-1 permissive cells in *trans*. DC-SIGN transfectants (100×10^6) were preincubated for 20 min at room temperature with blocking mAb against DC-SIGN (AZN-D1 and AZN-D2; 20 μg/ml). The THP-DC-SIGN cells were infected with HIV-luciferase virus pseudotyped with R5 strain $HIV\text{-}1_{JRA}$ Env. Alternatively activated T cells were infected with pseudotyped $HIV\text{-}1_{JRA}$ virus. After 2 hr at 37°C, the infected cells were extensively washed and added to either 293T-CD4-CCR5 cells or activated primary T cells (100×10^3). HIV-1 infection was determined after 2 days by measuring the luciferase activity. One representative experiment out of three is shown.

(B) DC-SIGN is able to mediate capture of HIV-1 viruses pseudotyped with M-tropic HIV-1 envelopes from different primary isolates. DC-SIGN-mediated capture was performed as described in (A) on 293T-CD4-CCR5 with HIV-luciferase viruses pseudotyped with the CCR5-specific HIV-1 envelopes from JRFL and JRCSF and from primary viruses 92US715.6, 92BR020.4, and 93TH966.8. One representative experiment out of two is shown.

tions after 2 days of coculture demonstrated that productive HIV-1 infection occured only in the HIV-1 permissive 293T-CD4-CCR5 cells (data not shown). Similarly, by using a pseudotyped HIV-1 vector with the green fluorescent protein gene in place of Nef (HIV-eGFP), we demonstrated that cells expressing CD4/CCR5 and not those expressing DC-SIGN were infected in cocultures. Thus, after coculture of virus-pulsed THP-DC-SIGN cells with T cells, only the $CD3^+$ T cells expressed virus-encoded GFP (Figure 4C).

Sexual transmission of HIV-1 is likely to require a means for small amounts of virus to gain access to cells that are permissive for viral replication. This may be achieved because of the ability of virus to interact with DC, which can capture HIV-1 and present it to the permissive cells. To mimic in vivo conditions in which HIV-1 levels are likely to be limiting, we challenged THP-1 transfectants with low titers of pseudotyped HIV-1 and subsequently cocultured these cells with HIV-1 permissive cells, without washing away unbound virus (Figure 5A). As expected, neither 293T-CD4-CCR5 cells nor activated T cells were efficiently infected with the low titers of pseudotyped HIV-1 (Figure 5A). Strikingly, when these permissive cells were challenged with an identical amount of HIV-1 in the presence of THP-DC-SIGN, but not of the parental THP-1 cells, efficient HIV-1 infection was observed in *trans* (Figure 5A). The enhancement of HIV-1 infection of primary T cells by DC-SIGN was also observed with HIV-luciferase viruses pseudotyped with five other R5 envelopes, including three from primary virus isolates (Figure 5B). These results indicate that DC-SIGN not only sequesters HIV-1 but also enhances CD4-CCR5-mediated HIV-1 entry by presentation in *trans* to the HIV-1 receptor complex. Antibodies against DC-SIGN completely inhibited infection (Figure 5A), demonstrating that the efficient enhancement of HIV-1 entry into CD4/CCR5-positive cells is DC-SIGN dependent.

DC Present in Mucosal Tissues at Sites of HIV-1 Exposure Express DC-SIGN and Are CCR5 Negative

Demonstration that cells that express DC-SIGN can capture HIV-1 and efficiently transmit the virus to other cells in *trans* suggested that DC that express this C-type lectin have a key role in viral infection in vivo. To determine whether such cells are indeed present in vivo, we performed immunohistochemical analyses of mucosal tissues that are the sites of first exposure during sexual transmission of HIV-1 (Figure 6A). DC-SIGN was expressed on DC-like cells with large and very irregular morphology that were present in the mucosal tissues, such as cervix, rectum, and uterus (Figures 6Aa, 6Ab, and 6Ac, respectively), in regions beneath the stratified

(C) Activated T cells are infected by HIV-1 in the T cell/THP-DC-SIGN coculture. THP-DC-SIGN cells were incubated with HIV-eGFP viruses pseudotyped with M-tropic $HIV\text{-}1_{JRA}$ and subsequently cocultured with activated T cells. The CD3-negative THP-DC-SIGN cells were not infected by HIV-1, whereas the CD3-positive T cells were infected. T cells, gated by staining for CD3 (tricolor), were positive for eGFP, whereas CD3-negative THP-DC-SIGN that initially captured HIV-eGFP did not express eGFP. One representative experiment out of two is shown.

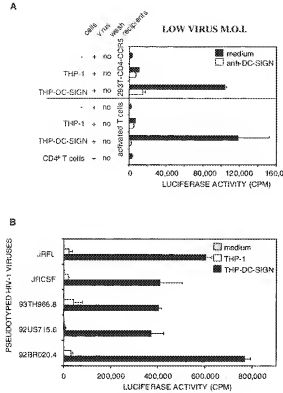


Figure 5. DC-SIGN Enhances HIV-1 Infection of T Cells by Acting In *trans*

At a low virus load, DC-SIGN in *trans* is crucial for the infection of HIV-1 permissive cells. THP-1 transfectants (100×10^3) were preincubated for 20 min at room temperature with blocking mAb against DC-SIGN (AZN-D1 and AZN-D2; 20 μg/ml). The cells were infected by low amounts of pseudotyped HIV-1_{BRL} virus (A) or other R5 isolates of HIV-1 (B), i.e., at the threshold of detection in a single round infection assay. After 1 hr at 37°C, the cell/virus suspension was directly added to either 293T-CD4-CCR5 or activated T cells (100×10^3). The infectivity was determined after 2 days by measuring the luciferase activity. One representative experiment out of two is shown.

squamous epithelium in the lamina propria. Analyses of serial sections stained for CD3, CD20, CD14, and CD68 confirmed that DC-SIGN-expressing cells were distinct from T cells, B cells, monocytes, and macrophages (data not shown). Similarly, in the accompanying paper (Geijtenbeek et al., 2000), staining of lymph nodes and skin has shown DC-restricted expression of DC-SIGN. We have also compared expression of DC-SIGN, CD4, and CCR5 on DC in the mucosa of the uterus and rectum and found in serial sections that the majority of DC-SIGN-positive DC in these tissues coexpressed CD4 but lacked CCR5 (Figure 6B). This suggests that DC present at mucosal sites, that have first contact with HIV-1 during sexual transmission, are not infected with HIV-1 through usage of CD4/CCR5. This observation is consistent with the recent demonstration that DC at sites of mucosal infection of nonhuman primates do not become infected (Stahl-Hennig et al., 1999).

DC-SIGN-Bound HIV-1 Retains Infectivity after Long-Term Culture

If HIV-1 gains access to secondary lymphoid organs by way of binding to DC, then virus would have to retain infectivity during the transport from the mucosal tissues to the T cell zones in draining lymph nodes. To determine if virus bound to DC-SIGN retains infectivity for a prolonged period of time, we first conducted a time-course experiment to determine the length of time that HIV-1 gp120 remains bound to DC-SIGN expressed on transfected THP-1 cells. We observed that gp120-coated beads remained bound to DC-SIGN for more than 60 hr (Figure 7A). We next investigated the length of time during which HIV-1-pulsed THP-DC-SIGN cells could retain infectious virus. The DC-SIGN-expressing transfectants were pulsed with pseudotyped HIV-1 for 4 hr and then washed extensively. The pulsed cells were subsequently placed in culture and were removed at defined intervals and cocultured with activated T cells (Figure 7B). Remarkably, after 4 days the HIV-1-pulsed cells were still able to efficiently infect target cells. In contrast, virus in the absence of DC-SIGN-positive cells lost its infectivity after 1 day. These findings support the hypothesis that limiting numbers of HIV-1 particles, captured by mucosal DC that express DC-SIGN and CD4 but not CCR5, retain infectivity during and after migration to regional lymphoid tissues. T cells, which express CD4 and CCR5, would then be productively infected due to DC-SIGN-mediated enhanced *trans* infectivity of the small numbers of HIV-1 particles (Figure 7C).

Discussion

We have identified a novel DC-specific adhesion receptor, DC-SIGN, that is identical to the high-affinity HIV-1 gp120-binding C-type lectin cloned from a human placental cDNA library (Geijtenbeek et al., 2000). We have demonstrated that DC that express both DC-SIGN and CD4 preferentially use DC-SIGN to capture HIV-1 via its high affinity for HIV-1 gp120. DC-SIGN not only efficiently recruits HIV-1 but also facilitates HIV-1 infection of $CD4^+$ T cells by a novel in *trans* mechanism. Our findings thus indicate that HIV-1 utilizes a novel receptor strategy that has not been previously described in other viral systems, and suggest that the virus exploits multiple cell surface receptor systems to ensure that it can establish a productive infection in its host organism.

DC localized in the skin and mucosal tissues such as the rectum, uterus, and cervix have been proposed to play a role in initial HIV-1 infection. DC constitute a heterogeneous population of cells that are present in minute numbers in various tissues just beneath the dermis or mucosal layer and form a first-line defense against viruses and other pathogens. DC have previously been shown to sequester HIV-1 and efficiently transmit the virus to $CD4^+$ T cells. We have demonstrated here that this property of DC can be ascribed to the ability of HIV-1 to bind specifically to these cells through the interaction of gp120 with DC-SIGN. DC thus efficiently capture HIV-1 through a specific interaction that is independent from binding of virus to CD4 and CCR5. DC-SIGN cannot mediate HIV-1 entry but rather functions as a unique HIV-1 *trans* receptor facilitating HIV-1 infection of CD4/CCR5-positive T cells (Figures 4 and 5). At low virus titer, CD4/CCR5-expressing cells are not detectably infected without the help of DC-SIGN in *trans* (Figure 5A). Conditions in which the number of HIV-1 particles is limiting are likely to resemble those

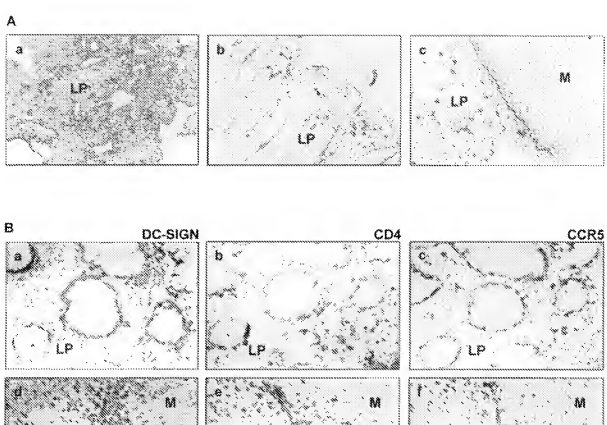


Figure 6. DC-SIGN Is Expressed on DC Present in Mucosal Tissue that Do Not Express CCR5

Immunohistochemical analysis of DC-SIGN expression on mucosal tissue sections.

(A) Different tissue sections were stained with anti-DC-SIGN mAb: cervix (a), rectum (b), and uterus (c) (original magnification, 200×) All mucosal tissues contain DC-SIGN-positive cells in the lamina propria. Staining of serial sections demonstrate that these DC-SIGN-positive cells do not express CD3, CD20, CD14, and CD68 (data not shown).

(B) Immunohistochemical staining of serial sections of rectum (a–c) and uterus (d–f) with antibodies against DC-SIGN (a and d), CD4 (b and e), or CCR5 (c and f).

found in vivo, and the results thus suggest that DC-SIGN may be required for viruses to be transmitted from mucosa to T cells that express CD4 and chemokine receptors. In addition, our studies demonstrate that virus bound to DC-SIGN is remarkably stable and can thus retain infectivity for the prolonged periods of time required for DC to traffic via lymphatics from mucosa to regional lymph nodes (Figures 7A and 7B).

Mechanism of DC-SIGN-Mediated Enhancement of HIV-1 Infectivity

The mechanisms by which HIV-1 exploits the machinery of DC and the properties of DC-SIGN to achieve efficient infection of cells that are competent for viral replication remain unclear. The process through which DC-SIGN promotes efficient infection in *trans* of cells through their CD4/chemokine receptor complex is of particular interest. Binding of the viral envelope glycoprotein to DC-SIGN may induce a conformational change that enables a more efficient interaction with CD4 and/or the chemokine receptor. As multiple conformational transitions are required before the envelope glycoprotein initiates fusion with target membranes, the binding of DC-SIGN to gp120 may facilitate or stabilize one of these transitions. Anti-gp120 antibodies that increase infectivity of viral particles have been described (Lee et al., 1997), and it is possible that DC-SIGN has a similar effect upon binding to the envelope glycoprotein. Alternatively, binding of viral particles to DC-SIGN may focus or concentrate them at the surface of the DC and may thus increase the probability that entry will occur after they bind to the receptor complex on target cells. Although the molecular mechanism has to be investigated in more detail, it is clear that DC-SIGN enhances the infection of T cells, since at low multiplicity of infection T cells are not infected in the absence of DC-SIGN.

Whether a transient quaternary complex is formed between DC-SIGN, HIV-1 Env, CD4, and CCR5 remains

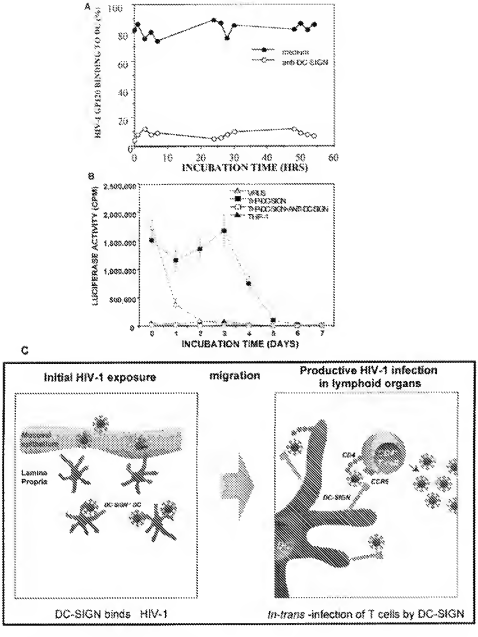


Figure 7. DC-SIGN Captures HIV-1 and Retains Long-Term Infectivity

(A) Time course of HIV-1_{IIIB} gp120 binding to THP-DC-SIGN. DC-SIGN-positive cells were incubated with gp120-coated beads. Beads bound for more than 60 hr as determined by FACScan analysis.

(B) DC-SIGN binds HIV-1 and retains for more than 4 days virus that infects T cells in *trans*. THP/THP-SIGN cells were pulsed for 4 hr with HIV-1 pseudotyped virus in the presence or absence of anti-DC-SIGN antibodies (AZN-D1 and AZN-D2, 20 μg/ml). After washing, the HIV-1 pulsed cells were cultured at 37°C for several days. As a control, identical amounts of virus were incubated at 37°C in medium without cells. Every day, aliquots of the HIV-1 pulsed cells were added to HIV-1 permissive 293T-CD4-CCR5 cells in order to measure infectivity. Lysates to examine luciferase activity were obtained after 2 days of coculture.

(C) Model of HIV-1 coopting DC-SIGN as a *trans* receptor after initial exposure. DC are the primary cells targeted by HIV-1 during mucosal exposure and are DC-SIGN positive. HIV-1 adheres to DC-SIGN via a high-affinity interaction, and the immature DC carrying HIV-1 migrates to the lymphoid tissues. Upon arrival, DC will cluster with T cells, and DC-SIGN enhances HIV-1 infection of T cells in *trans* leading to a productive and sustained infection.

to be determined. Elucidation of the crystal structure of a gp120-CD4 complex has revealed that most glycosylation sites within gp120 reside in a ridge that flanks the CD4-binding pocket (Kwong et al., 1998). Since mannan blocks the binding of gp120 to DC-SIGN, it is likely that this C-type lectin binds to one or more carbohydrate moieties in gp120. It remains possible, however, that the lectin domain of DC-SIGN interacts with the polypeptide

backbone of gp120. Further studies with mutant forms of gp120 and with soluble DC-SIGN may be informative in efforts to elucidate the mechanism of enhanced infectivity in *trans*.

In a separate study, we have shown that DC-SIGN binds to ICAM-3, which is expressed constitutively on the surface of T lymphocytes (Geijtenbeek et al., 2000). Enhancement of target cell infectivity by DC-SIGN-bound HIV-1 was not dependent on the presence of ICAM-3 on target cells. However, we observed that enhancement of infectivity was consistently better when target cells were T cells rather than 293-CD4-CCR5 cells. It remains possible that the efficiency of viral transmission from carrier DC to target T cells may also be enhanced by specific adhesive interactions other than DC-SIGN-ICAM-3, such as LFA-1-ICAM-1, which predominates the adhesion between DC and activated T cells (Geijtenbeek et al., 2000). Therefore, antibodies against DC-SIGN do not inhibit the DC-T cell transmission of HIV-1 postinfection (Figure 2C).

Role of DC in HIV Infection In Vivo

The only HIV-1 receptors previously known to have a role in HIV-1 entry were CD4 and a subset of the G protein–coupled chemokine receptors, including CCR5 and CXCR4. CCR5 functions as the major receptor for strains of virus previously classified as "macrophage-tropic," and only those strains that can utilize this chemokine receptor can be efficiently transmitted between individuals (Littman, 1998). Other gp120-binding receptors had been previously identified, including DC-SIGN and galactosyl ceramide (Harouse et al., 1991), but these had not been shown to be involved in viral entry. This study shows that DC-SIGN not only binds HIV-1 but can also sequester it and catalyze its entry into cells that express CD4 and chemokine receptors. Although it remains to be determined whether DC-SIGN has a significant role in HIV-1 pathogenesis in vivo, our in vitro results and the pattern of expression of the different receptors in mucosal tissues are consistent with its having a key function in the early stages of viral infection. Remarkably, our immunohistochemical analyses clearly demonstrate that CCR5 is not expressed in the lamina propria of HIV-1-related mucosal tissue (Figure 6), whereas DC-SIGN is abundantly expressed. This observation confirms and extends the findings of Hladik et al. (1999), who showed that DC present in the genital tract also lack CCR5, and strongly suggest that HIV-1 cannot infect DC present at mucosal sites.

DC-SIGN may therefore play a crucial role in initial HIV-1 exposure by mediating viral binding to DC present in mucosal tissues, rather than infection of these cells. The high level of expression of DC-SIGN on immature DC and its high affinity for gp120, which exceeds that of CD4 (Curtis et al., 1992), indicate that DC-SIGN is endowed with the ability to efficiently capture HIV-1, even when the virus is present in minute amounts. HIV-1 may subsequently exploit the migratory capacity of the DC to gain access to the T cell compartment in lymphoid tissues. DC must be activated to commence their migration, and it is hence possible that multimerization of DC-SIGN on the cell surface of DC by interaction with the multivalent virus particles may initiate this process. Interestingly, the time course experiment shows that DC-SIGN is able to capture and bind to HIV-1 for more than 4 days, after which the virus can still infect permissive cells. This long-term preservation of HIV-1 in an infectious state would appear to allow sufficient time for it to be transported by DC trafficking from mucosal surfaces to lymphoid compartments, where virus can be transmitted (Figure 7C) (Steinman et al., 1997). Several groups have reported that DC can migrate from the periphery to draining lymph nodes within 2 days after antigen exposure or HIV-1 challenge (Barratt-Boyes et al., 1997; Stahl-Hennig et al., 1999). Viral particles have been reported within endocytic vesicles of DC. This observation suggests that DC-SIGN-bound HIV-1 may be internalized and protected during the time required for the cells to complete their journey to the regional lymph nodes. Further studies will be required to determine if viral internalization is essential for maintenance of infectivity.

Our data suggest that, after HIV-1 has been ferried by DC to the lymphoid compartment, DC-SIGN presents the bound viral particles to the CD4/CCR5 complex present on T cells and greatly enhances their entry into these cells (Figure 7C). We showed that monoclonal antibodies directed against DC-SIGN blocked productive infection occurring in the T cell cocultures with CD4/CCR5-positive monocyte-derived DC. Therefore, even in the presence of obligatory HIV-1 receptors present in *cis* on target cells, DC-SIGN functions as a *trans* receptor for HIV-1 infection of T cells and is critical in the primary cocultures. This is an important example of how a receptor can work in *trans*. Interestingly, CD4 can facilitate HIV-1 infection of CD4-negative cells that express CCR5 by a *trans* receptor mechanism, although it remains unclear whether this is an important route of infection in vivo (Speck et al., 1999). In that case, interaction of envelope glycoprotein with CD4 results in a conformational change that permits binding of the virus to CCR5 on CD4-negative cells. Together with the results presented here, these studies indicate that HIV-1 can use receptors in *trans* to facilitate infection of cells that otherwise may be difficult to infect either because of lack of proper receptors or because of their anatomical distribution relative to the sites of HIV-1 exposure.

The discovery of the role of DC-SIGN in HIV-1 infection may have significant implications for understanding the mechanism of HIV-1 transmission and for developing strategies to prevent or block viral infection. The observation that transmission of infection is confined to R5 strains of HIV-1 has remained a major enigma. In preliminary studies, we found that DC-SIGN captures and enhances infection of both X4 and R5 strains, and it is thus unlikely that preferential interaction of DC-SIGN with CCR5 would account for the restriction in tropism during transmission. Nevertheless, the demonstration that uninfected DC contribute to the process of viral entry raises the possibility that the requirement for CCR5 utilization may reflect a requirement for interaction of multiple cell types. The inhibition of HIV-1 infection observed in the presence of anti-DC-SIGN antibodies suggests that interfering with the gp120-DC-SIGN interaction either during the capture phase of DC in the mucosa or during DC/T cell interactions in lymphoid organs could inhibit dissemination of the virus. Small molecule inhibitors, potentially carbohydrate-based, that block

the ability of gp120 to bind to DC-SIGN may be effective in prophylaxis or therapeutic intervention. Vaccine strategies aimed at eliciting mucosal antibodies that inhibit gp120 binding to DC-SIGN may also be efficacious in preventing early establishment of infection. The efficacy of gp120 vaccines has been measured as a function of the levels of neutralizing antibodies that inhibit HIV entry through CD4 and CCR5. Our results thus suggest that levels of antibodies that block virus binding to DC-SIGN or the DC-SIGN-mediated enhancement of infection may also be predictive of protection.

Experimental Procedures

Antibodies

The following mAb were used: 2D7 (anti-CCR5; Becton Dickinson and Co., Oxnard, CA) and CD4 (RPA-T4; PharMingen, San Diego, CA). Anti-DC-SIGN mAb AZN-D1 and AZN-D2 were obtained by screening hybridoma supernatants of human DC-immunized BALB/c mice for the ability to block adhesion of DC to ICAM-3, as measured by the fluorescent bead adhesion assay.

Cells

Immature DC were cultured as previously described (Geijtenbeek et al., 2000). Stable THP-1 transfectants expressing DC-SIGN were generated by transfection of THP-1 cells with pRc/CMV-DC-SIGN by electroporation similarly as described (Lub et al., 1997).

Fluorescent Bead Adhesion Assay

Carboxylate-modified TransFluorSpheres (488/645 nm, 1.0 μm; Molecular Probes, Eugene, OR) were coated with M-tropic HIV-1_{WM} envelope glycoprotein gp120 similarly as was described for ICAM-1 beads (Geijtenbeek et al., 1999). Streptavidin-coated beads were incubated with biotinylated F(ab')2 fragment rabbit anti-sheep IgG (6 μg/ml; Jackson Immunoresearch) followed by an overnight incubation with sheep-anti-gp120 antibody D7324 (Aalto Bio Reagents Ltd., Dublin, Ireland) at 4°C. The beads were washed and incubated with 250 ng/ml purified HIV-1 gp120 (provided by Immunodiagnostics, Inc., through the NIH AIDS Research and Reference Reagent Program) overnight at 4°C. The fluorescent beads adhesion assay was performed as described by Geijtenbeek et al. (1999).

HIV-1 Infection of Both DC and DC-SIGN Transfectants

The M-tropic strain HIV-1_{BaL} was grown to high titer in monocyte-derived macrophages (MDM). Seven days after titration of the virus stock on MDM, $TCID_{50}$ was determined with a p24 antigen ELISA (Diagnostics Pasteur, Marnes la Coquette, France) and estimated as 10^9/ml. DC (5×10^4) preincubated with mAb against DC-SIGN (AZN-D1 and AZN-D2) or CD4 (RPA-T4) (20 μg/ml) or a combination of CCR5-specific chemokines (RANTES, MIP-1α, MIP-1β; each 500 ng/ml) for 20 min at room temperature were pulsed for 2 hr with HIV-1_{BaL} (at a multiplicity of infection of 10^5 infectious units per 10^5 cells), washed, and cocultured with activated PBMC (50×10^4). No DC-T cell syncytium formation was observed. The postinfection experiment was performed similarly except that the mAb or chemokines were added after the washing step of the HIV-1 pulse, together with the activated PBMC. Culture supernatants were collected at day 5, 6, 7, and 9 after DC-T cell coculture and p24 antigen levels, as a measure of HIV-1 production were determined by a p24 antigen ELISA. PBMC were activated by culturing them in the presence of IL-2 (10 U/ml) and PHA (10 μg/ml) for 2 days.

Pseudotyped viral stocks were generated by calcium-phosphate transfections of 293T cells with the proviral plasmid pNL-Luc-E R (containing a luciferase reporter gene) or the proviral pHIV-eGFP (containing a GFP reporter gene) and expression plasmids for ADA, JRFL, and JRCSF gp160 envelopes. The isolation, identification, and construction of the plasmids encoding the primary virus envelopes from 92US715.6, 92BR020.4, and 93TH966.8 has been previously described (Bjorndal et al., 1997). Viral stocks were evaluated by limiting dilution on 293T-CD4-CCR5 cells. HIV-1 pseudotyped with murine leukemia virus (MLV), amphotropic Env, and vesicular stomatitis virus glycoprotein (VSV-G) were used to ensure target cell viability.

Immunohistochemical analyses were performed as described previously (Geijtenbeek et al., 2000).

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C-type Lectins L-SIGN and DC-SIGN Capture and Transmit Infectious Hepatitis C Virus Pseudotype Particles*

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The molecular mechanisms involved in the hepatic tropism of hepatitis C virus (HCV) have not been identified. We have shown previously that liver-expressed C-type lectins L-SIGN and DC-SIGN bind the HCV E2 glycoprotein with high affinity (Lozach, P. Y., Lortat-Jacob, H., de Lacroix de Lavalette, A., Staropoli, I., Foung, S., Amara, A., Houles, C., Fieschi, F., Schwartz, O., Virelizier, J. L., Arenzana-Seisdedos, F., and Altmeyer, R. (2003) *J. Biol. Chem.* 278, 20358–20366). To analyze the functional relevance of this interaction, we generated pseudotyped lentivirus particles presenting HCV glycoproteins E1 and E2 at the virion surface (HCV-pp). High mannose *N*-glycans are present on E1 and, to a lesser extent, on E2 proteins of mature infectious HCV-pp. Such particles bind to both L-SIGN and DC-SIGN, but they cannot use these receptors for entry into cells. However, infectious virus is transmitted efficiently when permissive Huh-7 cells are cocultured with HCV-pp bound to L-SIGN or to DC-SIGN-positive cell lines. HCV-pp transmission via L-SIGN or DC-SIGN is inhibited by characteristic inhibitors such as the calcium chelator EGTA and monoclonal antibodies directed against lectin carbohydrate recognition domains of both lectins. In support of the biological relevance of this phenomenon, dendritic cells expressing endogenous DC-SIGN transmitted HCV-pp with high efficiency in a DC-SIGN-dependent manner. Our results support the hypothesis that C-type lectins such as the liver sinusoidal endothelial cell-expressed L-SIGN could act as a capture receptor for HCV in the liver and transmit infectious virions to neighboring hepatocytes.

Hepatitis C virus (HCV),[1] a member of the *flaviviridae* and genus *Hepacivirus*, is essentially transmitted during parenteral exposures to infected material such as contaminated blood or needles. How HCV reaches hepatocytes, the principal site of virus replication, is not clear. Several cellular proteins have been proposed to act as receptors for HCV on hepatocytes including CD81, scavenger receptor class B, type I, and the low density lipoprotein receptor (1–3), but these cellular proteins are not sufficient to mediate viral entry into target cells (4). Furthermore, they are expressed on several cell types *in vivo* and therefore cannot explain the hepatic tropism of HCV. HCV in blood can diffuse freely into the liver. However, it is likely that liver sinusoidal endothelial cells (LSEC) represent an obstacle to passive diffusion from the blood into the hepatic tissue. LSEC prevent access of leukocytes to hepatocytes (5) and limit the passage of molecules larger than 12 nm in diameter from the sinusoidal lumen to hepatocytes (6). Thus, passive diffusion through the fenestrated liver endothelium seems unlikely to mediate rapid and efficient hepatocyte targeting by HCV (mean diameter superior to 50 nm). LSEC seem to play a role in the capture and concentration of hepatotropic viruses such as Duck Hepatitis B virus before their transmission to neighboring hepatocytes (7). Duck Hepatitis B virus whose envelope proteins are glycosylated (8) can be detected in the LSEC of infected ducks several hours after infection. C-type lectins-like L-SIGN might be the receptor responsible for liver-specific retention of enveloped hepatotropic viruses. L-SIGN is highly expressed in LSEC (9, 10) but not in hepatocytes. Furthermore, the related molecule, DC-SIGN, is detected in the liver in Kupffer cells (11), which are immobile liver macrophages localized close to LSEC and hepatocytes.

L-SIGN and DC-SIGN act as pathogen-recognition receptors (12) and share 77% sequence homology. The principal characteristic of these C-type lectins is that they interact with mannose residues of glycoproteins in a calcium-dependent manner via their C-terminal carbohydrate recognition domain (CRD) (13, 14). Several motifs localized in their cytoplasmic domain could be involved in the internalization of the lectin by endocytosis and its recycling toward the plasma membrane (12). Several viruses including HCV bind to these lectins via their envelope glycoproteins (15–17). Recently, DC-SIGN has been shown to mediate the infection of dendritic cells (DC) and a DC-SIGN expressing cell line by the Dengue virus, another member of the *flaviviridae* and *Flavivirus* (18, 19).

HCV E1 and E2 surface glycoproteins are naturally retained in the endoplasmic reticulum via their transmembrane domains and carry 6 and 11 high mannose *N*-glycans, respectively (20–22). Previously, we and others (23–25) reported that the HCV glycoproteins and virions contained in the sera of HCV patients were captured by L-SIGN and DC-SIGN. The high mannosylated glycoform of HCV E2 binds to L-SIGN and

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[1] The abbreviations used are: HCV, hepatitis C virus; LSEC, liver sinusoidal endothelial cells; mAb, monoclonal antibody; HIV, human immunodeficiency virus; GFP, green fluorescent protein; pp, pseudoparticles; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LS, L-SIGN; DCS, DC-SIGN; DMJ, 1-deoxymannojirimycin hydrochloride; VSVG, vesicular stomatitis virus G protein; CRD, C-terminal carbohydrate recognition domain; E1, envelope glycoprotein 1; E2, envelope glycoprotein 2; CMV, cytomegalovirus; PBMC, peripheral blood mononuclear cells; FACS, fluorescence-activated cell sorter; PNGase F, peptide *N*-glycosidase F; Endo H, endoglycosidase H; RLU, relative light units; DC, dendritic cells; m.o.i., multiplicities of infection.

DC-SIGN with high affinity (k_d = 6 and 3 nM, respectively) (23). However, due to the lack of an efficient culture system and purified HCV virions, no information is available on the glycosylation status of E1 and E2 on mature infectious HCV virions or on the functional relevance of these interactions.

In this study, we investigated the role of L-SIGN and DC-SIGN in viral entry and transmission to hepatocytes using pseudotyped lentivirus particles carrying functional E1 and E2 glycoproteins (26–28). Our results show that HCV-pp can interact with L-SIGN or DC-SIGN in a different manner compared with other viruses and that L-SIGN may act as a tissue-specific capture receptor for HCV.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Murine monoclonal antibodies (mAb) A4, A11, and H52 are directed against linear epitopes of E1 (A4) and E2 (A11 and H52) glycoproteins. Polyclonal human antibody New Lav Blot is directed against all human immunodeficiency virus (HIV) proteins including the p24 core protein. Phycoerythrin-conjugated murine mAbs anti-DC-SIGN (FAB161P), anti-L-SIGN (FAB162P), and anti-L-SIGN/anti-DC-SIGN (FAb1621P) were purchased from R&D Systems. Murine mAbs anti-L-SIGN/anti-DC-SIGN (mAb1621) and anti-L-SIGN (mAb162) were also purchased from R&D Systems. 1B10 is a DC-SIGN murine mAb directed against a conformational epitope of the DC-SIGN CRD and was described previously (16). Fluorescein isothiocyanate-conjugated mouse mAb anti-CD14 (MφP9) and anti-CD1a (H1149) were purchased from BD Biosciences. As controls, mouse isotypic antibodies IgG2a, IgG2b, IgG1-fluorescein isothiocyanate, IgG2b-fluorescein isothiocyanate, and IgG2b-phycoerythrin were used.

Envelope Proteins, Packaging, and Transfer Vector Constructs—The p8.71 HIV packaging construct, encoding the HIV gag and pol genes, and the pTrip-GFP and pTrip-luciferase plasmids, encoding an HIV-based transfer vector containing a CMV-GFP or a CMV-luciferase internal transcriptional unit, respectively, were described previously (Fig. 1A) (29–31). The pCDNA3-VSVG (vesicular stomatitis virus G protein) and phCMV-cE1E2 expression vectors encode the VSVG protein and the E1 and E2 glycoproteins from a HCV 1a genotype, respectively (Fig. 1A) (26). The plasmid coding for the *env* gene of the JR-cerebral spinal fluid isolate was used to produce HIV pseudoparticles (HIV-pp) (32). It codes for an HIV genome in which the Nef gene has been replaced by a luciferase reporter gene.

Cell Lines and C-type Lectin Expression—Huh-7 is an adherent human hepatocytic cell line, and HEK293T is a human kidney cell line. Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. BTHP1 cells are non-adherent human Raji B cells, previously misidentified as THP1 cells (33). BTHP1 cells expressing L-SIGN (BTHP1-LS) were described previously (34). Briefly, the cDNA coding for L-SIGN (NCBI accession number AF290886) was amplified by PCR from a normal pTrip vector expressing L-SIGN. The pCDNA3-L-SIGN (DC-SIGNR) sequence was digested by BamHI (5′) and XhoI (3′) and inserted into the pTrip vector. BTHP1, BTHP1-LS, and BTHP1 expressing DC-SIGN (BTHP1-DCS) cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin. All of the products used for cell culture were from Invitrogen.

Purification of Human Peripheral Blood Mononuclear Cells (PBMC) and Dendritic Cells—Human PBMC were isolated from healthy donors by density gradient centrifugation using Ficoll (Amersham Biosciences). Monocytes were negatively selected with magnetic beads (cated with a mixture of antibodies (Miltenyi Biotec). They were seeded at 10^6 cells·ml^{-1} and subsequently cultured in fresh RPMI 1640 medium supplemented with 10% FCS, 1% penicillin/streptomycin, 50 ng·ml^{-1} recombinant human interleukin-4 (PeproTech), and 800 units·ml^{-1} recombinant human granulocyte macrophage colony stimulating factor (Schering-Plough) for 6 days. Differentiation of dendritic cells (DC) was assessed by FACS analysis using CD14 down-regulation and CD1a up-regulation as parameters. For enhancement assays, isolated PBMC were cultured in RPMI 1640, 10% FCS medium containing phytohemagglutinin (3 μg·ml^{-1}, Murex) and interleukin-2 (10 units·ml^{-1}, Chiron) for 3 days as previously described (32). They were then seeded at 10^6 cells·ml^{-1} and subsequently cultured in fresh RPMI 1640 medium containing interleukin-2 (10 units·ml^{-1}).

Production and Purification of Pseudotyped Lentivirus Particles—HEK293T cells were seeded 1 day before transfection at 2.5×10^6 cells in a 10-cm plate in 10 ml of DMEM, 10% FCS. Medium was replaced 3 h before transfection by fresh medium (10 ml). Cells were transfected using a standard phosphate calcium transfection method (Clontech). The transfecting DNA mixture (1 ml) was composed of 8 μg of p8.71, 8 μg of pTrip-GFP or pTrip-luciferase, and 5 μg of glycoprotein(s) encoding plasmid. HCV-pp and VSV-pp correspond to lentivirus particles pseudotyped with HCV E1 and E2 glycoproteins and VSVG glycoprotein, respectively (Fig. 1*A*). For Δenv-pseudotyped lentivirus particles (Δenv-pp), the amount of envelope-encoding plasmid was replaced by the same amount of p8.71 plasmid (13 μg total). Medium was replaced 16 h after transfection by DMEM supplemented with 10% FCS (8 ml). To produce HCV-pp carrying high mannose *N*-glycans (HCV-pp^{DM1}), α-mannosidase I and II inhibitors 1-deoxymannojirimycin hydrochloride (DMJ, 1 mM, Calbiochem) and swainsonine (5 mM, Sigma) were added. For the production of ^{35}S-labeled HCV-pp, the medium was supplemented with 200 μCi·ml^{-1} [^{35}S]cysteine and methionine (Pro-Mix ^{35}S, Amersham Biosciences). HCV-pp were harvested in culture supernatants 24 h after transfection. Supernatants were cleared by centrifugation and filtered through 0.45-μm pore-sized membranes. HCV-pp were purified by ultracentrifugation through a 2.5-ml 20% sucrose cushion in a Beckman SW 28 rotor (28000 rpm; 2 h, 30 min; 4 °C). Virus was concentrated 100 times in FCS-free DMEM, and the p24 antigen content was determined with a commercial enzyme-linked immunosorbent assay (PerkinElmer Life Sciences).

Immunodetection of Cell Surface Antigens—L-SIGN and DC-SIGN were detected by FACS using phycoerythrin-conjugated anti-L-SIGN (FAb162P), anti-DC-SIGN (FAb161P), and anti-L-SIGN/anti-DC-SIGN (FAb1621P) antibodies. CD14 and CD1a were detected using fluorescein isothiocyanate-conjugated anti-CD14 (MφP9) and anti-CD1a (H1149). Cells were washed in FCS-free DMEM and resuspended in A buffer (1% bovine serum albumin, 0.2% γ-globulin, and 0.1% sodium azide (all from Sigma)) followed by incubation with antibodies at a 1:100 dilution for 30 min at 4 °C. Cells were washed and fixed with paraformaldehyde (3.2%, Electron Microscopy Sciences) prior to FACS analysis (FACSCalibur, BD Biosciences) and data-processed with CellQuest software (BD Biosciences).

Glycosylation of HCV-pp Proteins—HCV-pp (6 μg of p24 equivalent) were submitted to a 20–60% sucrose density gradient (10 ml) using a Beckman SW 41 rotor (40,000 rpm; 18 h; 4 °C). Fractions (500 μl) were collected and analyzed (18 μl) directly by Western blotting.

Purified HCV-pp or HCV-pp^{DM1} extracts (200 ng of p24 equivalent) were denatured in 0.5% SDS and 1% β-mercaptoethanol at 100 °C for 5 min followed by an overnight incubation at 37 °C in G7 buffer (50 mM sodium phosphate, pH 7.5, New England Biolabs), Nonidet P-40 buffer (1% Nonidet P-40, New England Biolabs) containing endoglycosidase H (5 milliunits of Endo H, Roche Applied Science) or peptide *N*-glycosidase F (1000 U of PNGase F, New England Biolabs). HCV-pp subjected to the same procedure in the absence of PNGase F or Endo H were used as a control.

Protein extracts were analyzed by SDS-PAGE (NuPAGE Novex Bis-Tris gels, Invitrogen) and transferred to Immobilon P membranes (Millipore). Incubation with primary antibodies A11 (anti-E2, 2 μg·ml^{-1}), H52 (anti-E2, 2 μg·ml^{-1}), A4 (anti-E1, 2 μg·ml^{-1}), and New Lav blot (anti-HIV, 1:200) was followed by incubation with an anti-mouse horseradish peroxidase-conjugated secondary antibody, NA931V (Amersham Biosciences) (1:1000). Bound antibodies were detected by exposure to enhanced chemiluminescence reagents (ECL+, Amersham Biosciences) and analyzed by a video acquisition system (Intelligent Dark Box II, Fuji) and Image Gauge software (Fuji).

Cell Infection with HCV-pp—Adherent cells (5×10^4) were plated in 12-well plates 24 h before infection in DMEM containing 10% FCS. 10^5 cells were washed three times with FCS-free medium, and 300 μl of diluted pseudotyped retroviral particles in FCS-free DMEM were added for 1 h at 37 °C under gentle agitation every 15 min without the addition of facilitating reagents such as DEAE-dextran or Polybrene. Subsequently, 300 μl of DMEM containing 10% FCS were added. 16 h later, viral inoculum was removed and replaced with 1 ml of DMEM supplemented with 10% FCS. Cells were harvested 52 h later. When GFP was used as the reporter gene, cells were fixed with paraformaldehyde (3.2%) and positive GFP cells were analyzed by FACScan (FACSCalibur, BD Biosciences). When luciferase was used as a reporter gene, cells were harvested in 150 μl of lysis B buffer (25 mM Tris phosphate, pH 7.8, 8 mM $MgCl_2$, 1 mM dithiothreitol, 1% v/v Triton X-100, 15% v/v glycerol) and 50 μl of lysis extract were measured in substrate B buffer containing 1 mM luciferin (Sigma) and 20 mM ATP (Sigma) in a Lumat LB 9501 (Berthold) to determinate the luciferase activity.

Binding of HCV-pp and HCV-pp^{DM1} to DC-SIGN and L-SIGN—Binding assays were performed on 10^6 BTHP1, BTHP1-LS, BTHP1-DCS, or dendritic cells in 200 μl of C buffer (RPMI 1640 medium without FCS containing 1 mM $CaCl_2$ and 2 mM $MgCl_2$) in 96-well plates.

Cells were incubated with ^{35}S-labeled HCV-pp (30 ng of p24 equivalent) for 3 h at 37 °C under gentle shaking. Virus-cell mixtures were then transferred to a new plate, and unbound radioactivity was removed by five washes with C buffer. Cell pellets were resuspended in C buffer prior to the addition of Optiphase Supermix solution (Wallac), and bound radioactivity was counted in a 1450 Microbeta Trilux γ counter (Wallac). For binding inhibition assays, cells were preincubated with inhibitors in C buffer for 30 min at 4 °C before the addition of labeled particles containing inhibitor. Inhibitors were used at a final concentration of 25 $\mu g ml^{-1}$ for mannan (Sigma), 1B10, MAb1621, and isotype control IgG or at 5 mM for EGTA (Sigma). HCV-pp-specific binding to BTHP1, BTHP1-DCS, BTHP1-LS, or dendritic cells was calculated as shown in Equation 1.

$$\text{Bound cpm} = \text{HCV-pp} - \text{Bound cpm from } \Delta\text{env-pp} \quad \text{(Eq. 1)}$$

HCV-pp and HCV-pp^{DMJ} Transmission by DC, BTHP1-LS, and BTHP1-DCS Cells—Huh-7 cells (5×10^4) were plated 1 day before transmission in 12-well plates in DMEM supplemented with 10% FCS. Before coculture, cells were washed three times with FCS-free medium and incubated in 300 μl of FCS-free DMEM. HCV-pp (150–300 ng of p24 equivalent) were incubated with BTHP1, BTHP1-LS, BTHP1-DCS, or dendritic cells (10^6) as described. Cells were resuspended in 300 μl of DMEM supplemented with 10% FCS and added to Huh-7 cells. Coculture was carried out for 68 h at 37 °C. Plates were washed three times followed by the harvesting and FACScan analysis or luciferase activity measurement as described. Virus transmission results were calculated as follows: 1% GFP(+) cells in coculture × 100/GFP(+) cells following direct infection with the same amount of HCV-pp and 2) RLU in coculture × 100/RLU following direct infection with same amount of HCV-pp.

Enhancement of HCV-pp and HIV-pp infection by L-SIGN and DC-SIGN were tested using Huh-7 and activated PBMC target cells, respectively. Huh-7 cells (2.5×10^4) were plated in 24-well plate in DMEM and 10% FCS 24 h before transmission. BTHP1, BTHP1-LS, BTHP1-DCS, or dendritic cells (5×10^4) were incubated with low and otherwise non-infectious quantities of HCV-pp, HCV-pp^{DMJ} (50 ng of p24 equivalent) or with HIV-pp as described previously (34) for 3 h at 37 °C. The DC, BTHP1, BTHP1-LS, and BTHP1-DCS virus mixture then was directly added to Huh-7 cells or activated human T lymphocytes cells (5×10^5). Co-culture was carried out for 68 h at 37 °C. Cells were washed three times prior to lysis in buffer B and luciferase activity measurement as described previously.

RESULTS

Characterization of Functional HCV-pp—To study the interactions between HCV and L-SIGN or DC-SIGN, we produced lentivirus-pseudotyped particles carrying unmodified HCV E1 and E2 glycoproteins (Fig. 1*A*) (26–28). The E1 and E2 lentivirus pseudotype particles, HCV-pp, were biologically functional because they could infect the hepatocytic cell line Huh-7 as evidenced by the expression of either a GFP (Fig. 1*B*) or a luciferase reporter gene (Fig. 1*C*). Non-infectious lentivirus viral particles lacking surface glycoprotein (Δenv-pp) were used as negative controls. Lentivirus particles carrying the G envelope protein of VSV were used as positive controls (VSV-pp).

HCV-pp were separated on a sucrose gradient, and fractions were collected and analyzed by Western blotting. The E1 protein was distributed in fractions in which neither E2 nor HIV core protein could be significantly detected (Fig. 2*A*). The fractions in which E1 protein was detected alone might correspond to lipid droplets carrying E1 protein. Infectious HCV-pp was present only in fractions where E1, E2, and HIV p24 core protein colocalized (Fig. 2*A*). No E1 or E2 protein was detected in Δenv-pp or supernatant of cells transfected with E1 and E2 protein coding plasmids alone (data not shown).

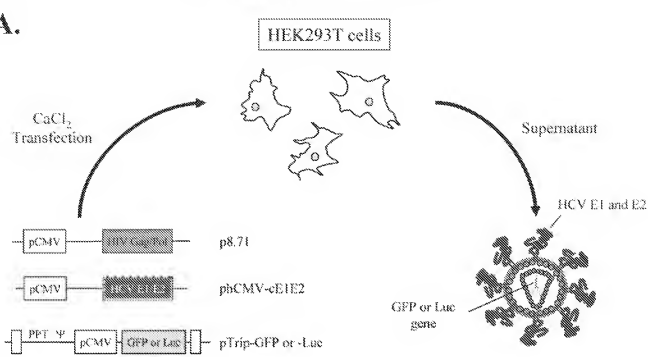
The subcellular compartment of HCV budding, *i.e.* where E1/E2 heterocomplexes interact with cores to form virus particles, and the presence of highly mannosylated or complex *N*-glycans on the HCV virion have not been determined. In agreement with a previous report (28), the HCV-pp-associated E2 is partially resistant to Endo H, indicating the *N*-glycan modification by Golgi-resident enzymes (Fig. 2*B*). To simulate a situation in which HCV *N*-glycans are not modified in the Golgi, we generated HCV-pp in the presence of swainsonine and DMJ, which inhibit Golgi α-mannosidases and therefore prevent complex glycosylation. The infectivity of these heavily mannosylated particles (HCV-pp^{DMJ}) in Huh-7 cells was similar to that of HCV-pp produced in the absence of swainsonine and DMJ (data not shown). As expected, HCV-pp^{DMJ} E2 glycoprotein remains completely sensitive to Endo H, demonstrating that it is highly mannosylated (Fig. 2*C*). Interestingly, E1 proteins on HCV-pp and HCV-pp^{DMJ} had the same glycosylation pattern (Fig. 2, *B* and *C*). We concluded that the upper band observed for E1 after Endo H treatment was a carbohydrate residue, which is not accessible to Endo H enzyme, and was not a complex carbohydrate residue. Our data suggest that during HCV-pp maturation, all of the E1 *N*-glycans and a minor fraction of E2 *N*-glycans remain inaccessible to glycosylation-modifying enzymes.

HCV-pp and HCV-pp^{DMJ} represent two glycosylation profiles of functional HCV E1 and E2 proteins that may be present on wild type virions. Therefore, HCV-pp and HCV-pp^{DMJ} are tools of choice to explore the biochemical correlations of a functional interaction of HCV with C-type lectin receptors.

HCV-pp and HCV-pp^{DMJ} Bind to L-SIGN and DC-SIGN—We previously reported that soluble HCV E2 protein binds to L-SIGN and DC-SIGN with high affinity (23). To study interactions between these lectins and functional E1 and E2 proteins on HCV-pp, L-SIGN and DC-SIGN were expressed on the surface of BTHP1 human cell line (BTHP1-LS and BTHP1-DCS, respectively) (Fig. 3*A*). Dendritic cells, which express DC-SIGN, were also differentiated from human PBMC. CD14 down-regulation and CD1a up-regulation confirmed the differentiation into DC (Fig. 8*A*). Δenv-pp and Δenv-pp^{DMJ} (produced in the presence of swainsonine and DMJ) did not bind to BTHP1 and BTHP1-LS but interacted weakly with BTHP1-DCS or dendritic cells. Δenv-pp (or Δenv-pp^{DMJ}), which does not incorporate viral glycoproteins, was used to define background values. Both ^{35}S-labeled HCV-pp and HCV-pp^{DMJ} bound to BTHP1-LS and BTHP1-DCS but not to control cells (Fig. 3*B*). Furthermore, DC-SIGN expressed on DC bound HCV-pp and HCV-pp^{DMJ} (Fig. 8*B*). However, at equimolar HIV p24, the core concentration of HCV-pp and HCV-pp^{DMJ}, a higher binding was obtained for HCV-pp^{DMJ}. This finding suggests that the presence of high mannose Endo H-sensitive glycosylation on HCV-pp^{DMJ} E2 protein enhances binding.

We sought to define further the specificity of these interactions by testing known inhibitors of ligand binding to DC-SIGN or L-SIGN. The CRD-specific monoclonal antibody 1B10 inhibited the binding of HCV-pp and HCV-pp^{DMJ} to DC-SIGN, and mAb1621 directed against DC-SIGN and L-SIGN inhibited the binding of HCV-pp and HCV-pp^{DMJ} to L-SIGN (Figs. 4, *A*, and *B*, and 8*B*). In addition, these interactions were blocked by the calcium chelator EGTA (Fig. 4, *A* and *B*), confirming the calcium-dependent binding of the CRD with high mannose *N*-glycans. In contrast, we did not observe inhibition by L-SIGN-specific mAb162 (R&D) (data not shown). Interactions of HCV-pp and HCV-pp^{DMJ} with L-SIGN and DC-SIGN thus are specific to envelope proteins E1 and E2 and are increased if E2 is highly mannosylated. These results suggest a pivotal role of high mannose *N*-glycans as binding motifs on HCV E2 protein.

Expression in Cis either of L-SIGN or DC-SIGN Does Not Enhance HCV-pp Infectiveness—L-SIGN and DC-SIGN are used by several viruses, including Dengue virus, to directly infect cells. No infection of BTHP1-LS, BTHP1-DCS (Fig. 5), or dendritic cells (Fig. 8*C*) by HCV-pp was observed, in agreement with a previous report (27). Similar results were obtained with HIV-pp or HCV-pp^{DMJ}, which displays increased binding to



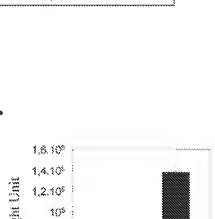


FIG. 1. **HCV E1/E2-pseudotyped lentivirus particles are biologically functional.** *A*, pseudotyped lentivirus particles were produced in HEK293T cells cotransfected with plasmids encoding HIV Gag/Pol proteins (p8.71), viral envelopes (phCMV-cE1E2 coding for HCV E1/E2 or pCDNA3-VSVG coding for VSVG protein) and a modified HIV RNA encoding the encapsidation signal (ψ) and a central GFP or luciferase transcriptional unit (pTrip-GFP or pTrip-Luc, respectively). *B*, GFP expression in HCV-pp- or VSV-pp-infected Huh-7 cells was detected by FACScan analysis. 10^5 cells were infected with 300 ng of p24 equivalent of HCV-pp. The data are representative of three independent experiments. *C*, luciferase activity in Huh-7 cells (10^5) infected by HCV-pp (300 ng of p24 equivalent). The data are expressed as RLU/mg of total protein and are representative of three independent experiments. Values are given as the mean of duplicates ± S.E.

both lectins (data not shown). Other cell lines expressing L-SIGN or DC-SIGN (HeLa and human osteosarcoma-derived cells) yielded similar results (data not shown). In contrast, these cells could be infected with Dengue virus as recently reported (data not shown) (18, 19). Retroviral particles carrying Dengue virus envelope proteins prM and E seem to act in a way similar to wild type Dengue virus (data not shown), substantiating the model of HCV-pp. Moreover, VSV-pp could infect these cells independently of the lectins. This led to the conclusion that a lack of permissiveness of BTHP1-LS or BTHP1-DCS

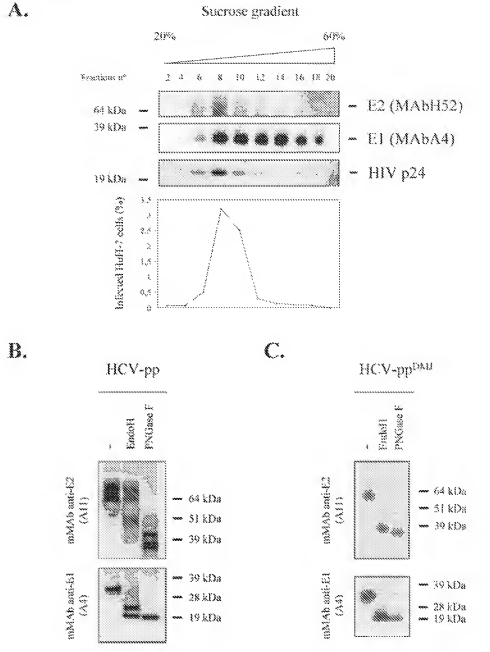


FIG. 2. **E1 and E2 glycoproteins on lentivirus particles carry high mannose *N*-glycans.** *A*, HCV-pp coding for GFP reporter gene were separated on a 20–60% sucrose gradient, and fractions were analyzed by Western blotting using E1-, E2-, and HIVp24-specific mAb and infectivity assays in Huh-7 cells. Optimal GFP expression was obtained in fractions where E1, E2, and HIV p24 core proteins colocalized. *B*, HCV-pp were subjected to digestion by Endo H or PNGase F and analysis by Western blotting. HCV-pp E2 proteins could be partially deglycosylated by Endo H and completely by PNGase F. *C*, HCV-pp produced in the presence of α-mannosidase inhibitors DMJ and swainsonine (HCV-pp^{DMJ}) were subjected to digestion by Endo H or PNGase F and analyzed by Western blotting. HCV-pp^{DMJ} E2 proteins were completely deglycosylated either by Endo H or by PNGase F. E1 protein of HCV-pp produced in the presence or absence of DMJ and swainsonine had the same glycosylation patterns.

cells to HCV-pp is because of a block at the level of virus entry. Hepatocytes and Huh-7 cells do not express L-SIGN and DC-SIGN. Expression of these lectins in these cells does not increase HCV-pp entry, whereas the infectivity of Huh-7 cells by VSV-pp remains unmodified (data not shown). Our results suggest distinct ways of DC-/L-SIGN usage by HCV and Dengue virus.

HCV-pp Captured by L-SIGN or DC-SIGN Are Transmitted to Hepatocytic Cells—We investigated whether HCV-pp captured by BTHP1-LS or BTHP1-DCS cells could be transmitted to the hepatocytic cell line Huh-7. Cells were incubated with HCV-pp (coding for the GFP or luciferase reporter gene), and unbound virus was eliminated by repeated washings prior to coculture with Huh-7 target cells. The transmission of HCV-pp captured by BTHP1-LS or BTHP1-DCS cells was confirmed with both reporter systems (Fig. 6). HCV-pp weakly interact with BTHP1 cells, because residual transmission could be observed in comparison with Δenv-pp. However, the HCV-pp transmission was on average 5-fold higher for BTHP1-LS or BTHP1-DCS compared with background transmission with BTHP1 (Fig. 6, *A* and *B*). Moreover, primary DC can efficiently transmit HCV-pp to Huh-7 cells (Fig. 8*D*). The transmission was specific, because it was inhibited by monoclonal antibodies MAb1621 and 1B10. EGTA also inhibited transmission of HCV-pp by BTHP1 cells expressing these lectins. HCV-pp transmission by DC-SIGN could also be completely inhibited by mannan, whereas transmission by L-SIGN was only weakly inhibited in the presence of mannan. The highest inhibition rate obtained for mannan was 40%. Interestingly, HCV-pp^{DMJ} carrying highly mannosylated proteins were transmitted with efficiency similar to that of HCV-pp carrying mixed glycosylation (Fig. 6*C*). The difference between HCV-pp and HCV-pp^{DMJ} resides in the modification of the E2 glycosylation pattern. Two non-exclusive hypotheses can be put forward to explain why we do not see increased transmission of HCV-pp^{DMJ}. First, a specific binding motif composed of one or more high mannose *N*-glycans could be present on E1 or E2 of HCV-pp, allowing optimal capture and transmission by DC-/L-SIGN, or second,

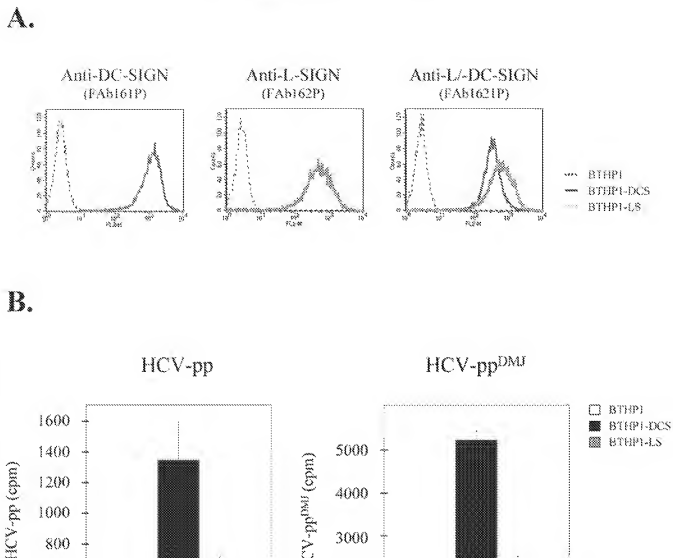


FIG. 3. **HCV-pp and HCV-pp^{DMJ} bind to C-type lectins L-SIGN and DC-SIGN.** *A*, expression of L-SIGN and DC-SIGN in BTHP1-LS and BTHP1-DCS cell lines, respectively, was detected by FACScan. *B*, ^{35}S-labeled HCV-pp and HCV-pp^{DMJ} bound to BTHP1-LS or BTHP1-DCS cells. Bound radioactivity for particles without viral envelope, Δenv-pp and Δenv-pp^{DMJ}, was subtracted from values obtained with HCV-pp and HCV-pp^{DMJ}. The data are representative of five independent experiments. Values are given as the mean of duplicates ± S.E.

the mechanism implicated in HCV-pp and HCV-pp^{DMJ} transmission is saturated in the experimental conditions used in this study.

DC-SIGN expressed *in trans* was previously shown to boost HIV infection of T cells at low multiplicities of infection (m.o.i.) (17, 34). Indeed, when we used low m.o.i. of HIV-pp, DC-SIGN on BTHP1 or dendritic cells, and to a lesser extent L-SIGN on BTHP1 cells, we were able to enhance HIV infection of activated T cells (Figs. 7 and 8*E*). To test whether DC-SIGN and L-SIGN could also enhance HCV-pp infectivity, DC-SIGN and L-SIGN BTHP1 cells or dendritic cells were preincubated with a low m.o.i. of HCV-pp and then cocultured with Huh-7 cells (Figs. 7 and 8*E*). Under these experimental conditions and in contrast to HIV-pp, no enhancement of viral infectivity was observed.

DISCUSSION

The expression of L-SIGN on liver sinusoidal endothelial cells and its capacity to bind high mannose *N*-glycans on viral glycoproteins make this lectin a candidate receptor responsible for liver tropism of HCV and other enveloped hepatotropic viruses. We established previously that a soluble form of the highly mannosylated HCV E2 envelope glycoprotein binds to L-SIGN and the related C-type lectin DC-SIGN with high affinity (23). In this report, we provide insight into the functional relevance of this interaction with respect to the

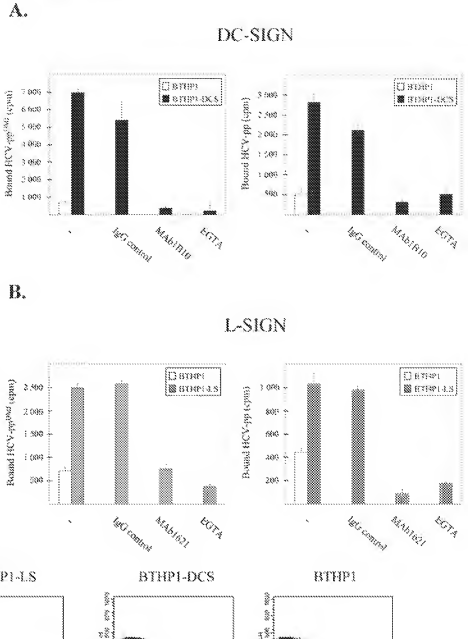


FIG. 4. **Specificity of HCV-pp and HCV-pp^{DM5J} binding to L-SIGN and DC-SIGN.** Inhibitors for C-type lectin binding were used in a binding assay with ^{35}S-labeled HCV-pp and HCV-pp^{DM5J}. *A*, specificity of binding to DC-SIGN. HCV-pp and HCV-pp^{DM5J} were incubated with BTHP1-DCS cells in the presence of EGTA (5 mM), mAb1B10 directed against the CRD of DC-SIGN (20 μg·ml^{-1}), or a control IgG2a (20 μg·ml^{-1}). *B*, specificity of HCV-pp and HCV-pp^{DM5J} binding to L-SIGN. HCV-pp and HCV-pp^{DM5J} were incubated with BTHP1-LS cells in the presence of EGTA (5 mM), mAb1621, which blocks L-SIGN (20 μg·ml^{-1}), or a control IgG2b (20 μg·ml^{-1}). Bound radioactivity for particles without viral envelope, Δenv-pp and Δenv-pp^{DM5J}, was subtracted from values obtained with HCV-pp and HCV-pp^{DM5J}. Values are given as the mean of duplicates ± S.E. The data are representative of two independent experiments.

FIG. 5. **L-SIGN and DC-SIGN do not mediate entry of HCV-pp into cells.** BTHP1-LS and BTHP1-DCS cells (10^5) were infected with HCV-pp or VSV-pp (300 ng of p24 equivalent). Expression of GFP reporter gene was detected by FACS 72 h post-infection. The data are representative of more than three independent experiments.

capture of HCV by both lectins and transmission to target cells.

Because of the lack of an *in vitro* system for growth, purification, and biochemical analysis of HCV virions, the molecular mechanisms of HCV particle formation and entry cannot be addressed using wild type HCV contained in serum from infected patients. Therefore, we generated functional lentivirus (HIV) particles in which the surface envelope glycoproteins

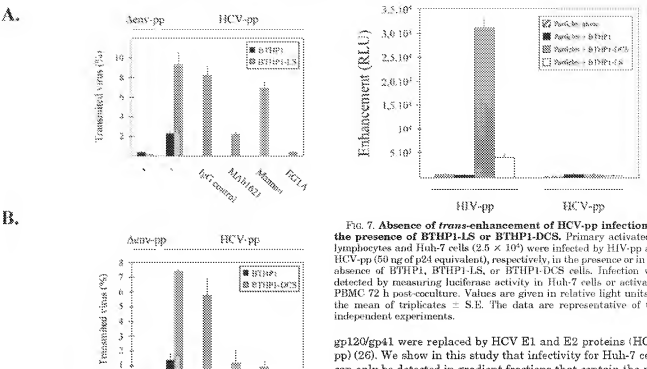


FIG. 6. **BTHP1-LS and BTHP1-DCS transmit captured HCV-pp to Huh-7 target cells.** Following binding of HCV-pp to BTHP1 cells, cells were washed five times prior to coculture with Huh-7 cells. Transmitted virus in the coculture was measured after 72 h of coculture using specific detection of a reporter gene. *A*, HCV-pp transmission by L-SIGN. Specificity was analyzed by adding the L-SIGN blocking antibody mAb1621 (20 $\mu g ml^{-1}$), EGTA (5 mM), and mannan (20 $\mu g ml^{-1}$) prior to HCV-pp binding. Values are given as the mean of quadruplicates ± S.E. The data are representative of three independent experiments and were obtained with HCV-pp encoding the luciferase reporter gene. The percentage of transmitted HCV-pp was determined as follows: (RLU in coculture × 100)/RLU for direct Huh-7 cells infection. The mean RLU value for directly infected Huh-7 cells was 9483 ± 2058. *B*, HCV-pp transmission by DC-SIGN. Specificity was analyzed by adding the DC-SIGN-blocking antibody 1810 (20 $\mu g ml^{-1}$), EGTA (5 mM) and mannan (20 $\mu g ml^{-1}$) prior to HCV-pp binding. Values are given as the mean of duplicates ± S.E. The data are representative of three independent experiments and were obtained with HCV-pp encoding a GFP reporter gene. The percentage of transmitted HCV-pp was determined as follows: (GFP(+) cells in coculture × 100)/GFP(+) cells for direct Huh-7 cells infection. The percentage of directly infected Huh-7 cells was 21.7% ± 0.2. *C*, HCV-pp^{DM3} transmission by L-SIGN and DC-SIGN. The capacity of transmission of HCV-pp^{DM3}, which have an increased binding capacity (Fig. 3*B*), was compared with that of particles lacking viral envelope glycoprotein (Δenv-pp). The percentage of transmitted HCV-pp and HCV-pp^{DM3} was determined as follows: (RLU in coculture × 100)/RLU for direct Huh-7 cells infection. The mean RLU values for directly infected Huh-7 cells were 4620 ± 90 and 3945 ± 576, respectively. Values are given as the mean of triplicates ± S.E. The data are representative of two independent experiments.

FIG. 7. **Absence of *trans*-enhancement of HCV-pp infection in the presence of BTHP1-LS or BTHP1-DCS.** Primary activated T lymphocytes and Huh-7 cells (2.5×10^5) were infected by HIV-pp and HCV-pp (50 ng of p24 equivalent), respectively, in the presence or in the absence of BTHP1, BTHP1-LS, or BTHP1-DCS cells. Infection was detected by measuring luciferase activity in Huh-7 cells or activated PBMC 72 h post-coculture. Values are given in relative light units as the mean of triplicates ± S.E. The data are representative of two independent experiments.

gp120/gp41 were replaced by HCV E1 and E2 proteins (HCV-pp) (26). We show in this study that infectivity for Huh-7 cells can only be detected in gradient fractions that contain the p24 HIV core and HCV E1 and E2 envelope proteins. These data are supported by previous studies that established that pseudotyped retroviral particles require both proteins for infection and that particles expressing either the E1 or the E2 protein do not enter target cells (26–28). Our data are in favor of a model proposing that E1 and E2 proteins are functional when associated as non-covalent heterodimers. Such heterodimers have been implicated in the native folding and maturation of the HCV envelope (35, 36). Biochemical analysis of infectious HCV-pp revealed an apparent dichotomy in glycosylation between E1 and E2. Although E1 *N*-glycans are not modified to complex Endo H-resistant *N*-glycans, E2 is almost completely Endo H-resistant, leaving only a minor fraction of *N*-glycans in a highly mannosylated state. The subcellular compartment where E1/E2 heterocomplexes are incorporated into HCV-pp is not known. This might occur in the endoplasmic reticulum or another compartment of the secretory pathway or at the plasma membrane. In the latter case, pseudotype particles incorporated E1/E2 heterodimers that have escaped endoplasmic reticulum retention and proceeded through the secretory pathway. In either setting, our results imply that some *N*-glycans in the E1/E2 heterodimer are inaccessible to sugar-modifying enzymes, resulting in the assembly of infectious HCV-pp that incorporate E1 and E2 proteins with several preserved high mannose *N*-glycans.

By analogy with other members of *flaviviridae*, it has been proposed that HCV buds into the endoplasmic reticulum. It is not known whether HCV subsequently passes through the secretory pathway and whether surface glycoproteins undergo modification of *N*-glycans from high mannosylated to complex in the Golgi. To simulate a high mannose E1/E2 glycosylation status of virus, we produced HCV-pp in the presence of α-mannosidase inhibitors (HCV-pp^{DM3}). HCV-pp^{DM3} carry Endo H-sensitive E2 and show similar infectivity in Huh-7 cells compared with HCV-pp, suggesting that com-

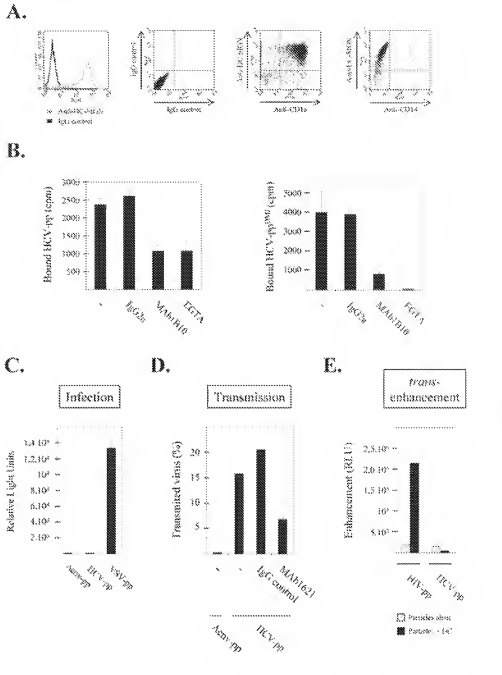


FIG. 8. **HCV-pp do not infect human primary dendritic cells but can be captured and transmitted to Huh-7 target cells.** *A*, expression of DC-SIGN in human primary DC by FACScan. CD1a up-regulation and CD14 down-regulation assess the differentiation of dendritic cells. *B*, ^{35}S-labeled HCV-pp and HCV-pp^{DMJ} bound to DC. HCV-pp and HCV-pp^{DMJ} were incubated with DC in the presence of EGTA (5 mM), DC-SIGN-blocking antibody mAb1621 (20 μg·ml^{-1}), or a control IgG2b (20 μg·ml^{-1}). Values are given as the mean of duplicates ± S.E. *C*, DC-SIGN does not mediate entry of HCV-pp into DC. Values are given as the mean of triplicates ± S.E. *D*, DC transmit captured HCV-pp to Huh-7 cells. Specificity was analyzed by adding mAb1621 (20 μg·ml^{-1}) or control IgG2b (20 μg·ml^{-1}) prior to HCV-pp binding. DC were washed and then cocultured with Huh-7 cells. Values are given as the mean of duplicates ± S.E. The percentage of transmitted HCV-pp was determined as follows: (RLU in coculture × 100/RLU for direct Huh-7 cells infection. The mean RLU value for directly infected Huh-7 cells was 43416 ± 974. *E*, primary activated T lymphocytes and Huh-7 cells (2.5×10^4) were infected by HIV-pp and HCV-pp (50 ng of p24 equivalent), respectively, in the presence or in the absence of DC. Infection was detected by measuring luciferase activity in Huh-7 cells or activated PBMC after 72 h of coculture. Values are given in RLU as the mean of triplicates ± S.E. The data presented in *panels A–E* are representative of two independent experiments with DC from two healthy blood donors.

plex *N*-glycans are not involved in the recognition of the HCV receptor(s) on Huh-7 cells.

Our binding studies show that infectious HCV-pp attaches to L-SIGN and DC-SIGN expressed at the plasma membrane. This interaction is specific and can be blocked by several lectin binding inhibitors. However, mannan can totally inhibit the HCV-pp transmission by DC-SIGN, whereas its effect on L-SIGN is poor. Such differences have been observed for Sindbis virus (37) and suggest that carbohydrate motifs recognized by L-SIGN and DC-SIGN are different.

In contrast to other enveloped viruses, the attachment of HCV-pp or HCV-pp^{DMJ} either to DC-SIGN or L-SIGN did not enhance entry into permissive cell lines or primary human DC-SIGN(+) DC. This finding is in contrast to a previous report showing that HCV-negative strand RNA, indicating replication of the viral genome, could be detected in DC (38). However, Goutagny *et al.* (39) report that positive strand viral RNA was associated with DC in HCV-infected patients but that only a small percentage of them had negative strand viral RNA associated with DC. Moreover, LSEC (40) or primary human Kupffer cell cultures (41) that express L-SIGN or DC-SIGN, respectively (9–11), are resistant to HCV infection. This stands in sharp contrast to the permissiveness to HCV demonstrated for the neighboring hepatocytes where negative strand RNA is readily detected (42). Whether DC-SIGN or L-SIGN mediates HCV endocytosis is unknown. Should this be the case, one could speculate that the lectin routes the virus to a subcellular compartment where HCV-induced membrane fusion cannot occur and does not lead to productive infection. Based on our current findings and the reported lack of HCV infection of DC-SIGN or L-SIGN expressing cells *in vivo*, we conclude that these lectins do not mediate infection in *cis* by HCV.

DC-SIGN enhances CMV and HIV infectivity of cells that are poorly permissive originally. This phenomenon was named "*trans*-enhancement." In the HIV model, DC-SIGN expressed

by DC-enhanced T cell infection at a low m.o.i. of the virus, whereas no infection was observed in its absence (17, 34, 45). Our results extend these findings for HIV to L-SIGN but more studies are required to characterize the observed efficiency difference between the two lectins. However, no enhancement was detected when low multiplicities of HCV-pp were used. Some specific cellular proteins may be critical for this mechanism, and their absence from Huh-7 cells may abrogate enhancement.

Whereas HCV-pp differ from other viruses with respect to *cis*-infection of DC-/L-SIGN expressing cells and to DC-/L-SIGN dependent *trans*-enhancement mechanisms, infectious HCV-pp can be captured by L-SIGN and DC-SIGN and transmitted to target cells as has been described for CMV (16), HIV (17), Ebola virus (15), and severe acute respiratory syndrome coronavirus (44). Indeed, our results show that coculture of the hepatocyte cell line Huh-7 with HCV-pp-loaded L-SIGN and DC-SIGN expressing cells results in productive infection of the Huh-7 cells. A model in which LSEC play a central role in the capture of hepatotropic viruses and their transmission to hepatocytes was recently proposed (7). Breiner *et al.* (7) show that the Duck Hepatitis B virus surface protein (preS) and virions preferentially had accumulated in LSEC *in vitro* and *in vivo* in infected ducks. No productive infection by Duck Hepatitis B virus was observed in LSEC, although particles seem to be internalized by endocytosis, which could lead to the creation of a viral reservoir. In the HCV setting, upon release from LSEC, the virus might use entry receptor(s), *e.g.* CD81, scavenger receptor class B, type I, low density lipoprotein receptor (1–3), heparan sulfate (45), or the asialoglycoprotein receptor (46) for entry and productive infection of neighboring hepatocytes. Our results are in favor of a model proposing LSEC and LSIGN, respectively, as cellular and molecular effectors of hepatotropism of enveloped viruses. Moreover, HCV could use DC-SIGN on DC as a transporter to assure its dissemination, similar to a mechanism proposed for HIV. Recent studies by Yoneyama (47) indicates that myeloid DC could migrate to the liver where DC-SIGN-positive cells have previously been detected (9, 10). It remains to be clarified whether HCV is internalized into L-SIGN- or DC-SIGN-expressing cells, *e.g.* LSEC or Kupffer cells, and whether virus endocytosis would be a prerequisite for efficient transmission to hepatocytes. Both L-SIGN and DC-SIGN encompass endocytosis motifs in the cytoplasmic N terminus, but their role on HCV-pp transmission needs to be analyzed. Internalized HCV in DC-SIGN- or L-SIGN-positive cells could function as a viral reservoir for infection of neighboring hepatocytes.

We propose that in the anatomical sites where HCV replicates, C-type lectins expressed on liver sinusoidal cells may act as the capture receptor for HCV and transmit infectious virions to neighboring hepatocytes. This search should be extended to LSECtin, another C-type lectin closely related to L-SIGN and specifically expressed in LSEC (48). DC-/L-SIGN might not be the sole molecule(s) implicated in the transmission of HCV or other hepatotropic viruses.

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C-Type Lectins DC-SIGN and L-SIGN Mediate Cellular Entry by Ebola Virus in *cis* and in *trans*

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Ebola virus is a highly lethal pathogen responsible for several outbreaks of hemorrhagic fever. Here we show that the primate lentiviral binding C-type lectins DC-SIGN and L-SIGN act as cofactors for cellular entry by Ebola virus. Furthermore, DC-SIGN on the surface of dendritic cells is able to function as a *trans* receptor, binding Ebola virus-pseudotyped lentiviral particles and transmitting infection to susceptible cells. Our data underscore a role for DC-SIGN and L-SIGN in the infective process and pathogenicity of Ebola virus infection.

Ebola virus is responsible for several major outbreaks of hemorrhagic fever, the exceedingly high mortality of which has raised great public concern. Ebola virus research has been hampered by the strict biosafety containment procedures required for handling the infectious agent. However, the structural similarity of Ebola virus glycoprotein (GP) to retroviral envelopes (6) has recently allowed the generation of pseudotyped recombinant retroviral particles that have been used to explore important aspects of the Ebola virus biology (16, 18). Ebola virus cell entry is presumably mediated by the interaction of a cellular receptor with the GP1 subunit of the viral envelope (12). A cofactor for cellular entry of Ebola virus and Marburg filoviruses in certain cell types has been recently identified as the folate receptor α (FRα) (3). This molecule is a glycophosphatidylinositol-linked protein highly conserved in mammalian species and expressed in epithelial and parenchymal cells of a number of organs, but not abundantly in liver or endothelial cells (15).

DC-SIGN (dendritic cell [DC]-specific ICAM-3 grabbing non-integrin, CD209) is a type II membrane protein with a C-type lectin extracellular domain, the expression of which is restricted to immature DC. DC-SIGN appears to play a key role in the initial stages of immune response and in the migratory behavior of DC, because it mediates DC interactions with T lymphocytes and endothelial cells through recognition of ICAM-3 (9) and ICAM-2 (7). DC-SIGN, originally cloned as a human immunodeficiency virus (HIV) gp120-binding protein (5), does not act as a receptor for cellular entry of HIV; instead, it confers to DC the ability to facilitate infection in *trans* of susceptible cells (8). Recently, DC-SIGN and the newly described DC-SIGN homologue L-SIGN have been shown to bind most lentiviruses of primates: HIV-1 (both R5 and X4 strains), HIV-2, and simian immunodeficiency virus (SIV) (13). Unlike DC-SIGN, L-SIGN is not expressed by DC, but is expressed on the surface of endothelial cells in the liver, lymph node sinuses, and placental villi (2). The affinity of these membrane receptors for retroviral GP and their tissue distribution pattern prompted us to study their potential role as binding and entry cofactors for Ebola virus.

To investigate the participation of DC-SIGN in Ebola virus infection, we have utilized lentiviral particles pseudotyped with Ebola virus GP according to a transient transfection protocol previously described (17). The lentiviral vector pNL4-3.Luc.R$^-$E^{-10} was used for production of vesicular stomatitis virus G (VSV-G) and Ebola virus Zaire and Reston GP pseudotypes. Expression plasmids for the GP of the Zaire and Reston strains of Ebola virus were kindly provided by A. Sanchez, Centers for Disease Control and Prevention (18). Supernatants were obtained 48 h after transfection, filtered (0.45-μm pore size), and stored frozen at −80°C. Infectious titers were estimated by serial dilution on HeLa cells and were typically in the range of 10^7 infectious units/ml for VSV-G and 10^5 infectious units/ml for Ebola virus GP pseudotypes. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute for Allergy and Infectious Diseases: DC-SIGN and L-SIGN monoclonal antibody DC28 (0.8 mg/ml as ascitic fluid) from F. Baribaud, S. Pöhlmann, J. A. Hoxie, and R. W. Doms (1); pcDNA3-L-SIGN6 from Mary Carrington; and pNL4-3.Luc.R$^-$E$^-$ from Nathaniel Landau (10).

To investigate the role of DC-SIGN in Ebola virus binding and cellular entry, we first used a stable transfectant of DC-SIGN in the erythroleukemic K562 cell line (14). K562 cells were incubated overnight in 24-well plates with supernatants containing Ebola virus GP-pseudotyped lentivirus at a multiplicity of infection (MOI) of 0.1. Infectivity was measured 48 h after infection by luciferase assay with reagents from Promega (Madison, Wis.) in a Berthold Sirius luminometer (Berthold, Munich, Germany) with a dynamic range from 10^2 to 10^7 relative light units (RLU). Infectivity of the parental K562 cells with an Ebola virus GP-pseudotyped lentiviral construction was detectable, although relatively low. In contrast, infectivity of the DC-SIGN transfectant cell line was 1 order of magnitude higher, and it was significantly reduced in the presence of

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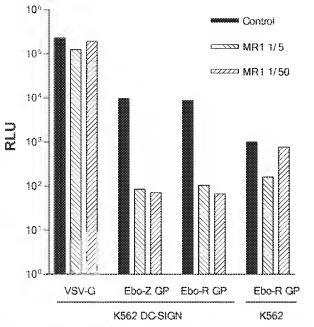

FIG. 1. DC-SIGN-mediated infection of K562-DC-SIGN cells. K562 and K562-DC-SIGN cells were infected with VSV-G-, Ebola virus Zaire (Ebo-Z)-, or Ebola virus Reston (Ebo-R) GP-pseudotyped lentivirus in the absence (control) or presence of the DC-SIGN-specific monoclonal antibody MR-1. Infectivity was measured as luciferase activity 48 h postinfection. One representative experiment out of three is shown.

the DC-SIGN-specific monoclonal antibody MR-1, thus suggesting that Ebola virus might interact with DC-SIGN and facilitate viral entry into K562-DC-SIGN-transfected cells (Fig. 1). MR-1 was used as tissue culture supernatant (10 μg/ml) and showed no reactivity with HeLa and K562 cells, as well as a panel of myeloid and lymphoid cell lines (14).

To further characterize the role of DC-SIGN and its close homologue L-SIGN in Ebola virus cell entry, we expressed, by using retroviral vectors, DC-SIGN and L-SIGN in the Jurkat cell line, since these cells are nonpermissive for Ebola virus infection and are considered receptor deficient (16). Recombinant retroviruses were produced as described previously (17) by cotransfection of the plasmids pNGVL-MLV-gag-pol and pCMV-VSV-G and the retroviral vector pLZRs-DC-SIGN-gfp—constructed by subcloning the DC-SIGN coding sequence obtained from placental RNA by reverse transcription-PCR with primers AAA AGG ATC CGC CGC CAC CAT GAG TGA CTC CAA GGA ACC (forward) and AAA AGA ATT CCT ACG CAG GAG GGG GGT TT (reverse), into the bicistronic retroviral vector pLZRs-M10-gfp (17), digested with *Bam*HI and *Eco*RI—or pLZRs-L-SIGN-gfp constructed in a similar way with the L-SIGN coding sequence obtained from pcDNA3-L-SIGN6. Plasmids pNGVL-MLV-gag-pol, pLZRs-RevM10-gfp, and pCMV-VSV-G were generously provided by G. Nabel, University of Michigan (17). Jurkat cells were transduced with VSV-G-pseudotyped DC-SIGN- or L-SIGN-expressing retroviral vectors by spinoculation for 2 h at 1,500 × *g* at an MOI of 10. After 48 h, cells were analyzed by fluorescence-activated cell sorting for green fluorescent protein (GFP) and lectin expression (range of positive cells, 10 to 30%) and challenged in 24-well plates with Ebola virus GP pseudotypes or controls: 250,000 cells were resuspended in 250 μl of complete medium (RPMI, 10% fetal bovine serum [FBS]) and incubated overnight with 250 μl of supernatant from transfections. Cells were assayed for luciferase expression 48 h postinfection. For inhibition experiments, cells were preincubated for 10 min at room temperature with the carbohydrate-interaction inhibitor mannan (25 μg/ml; Sigma, St. Louis, Mo.) or lectin-specific antibodies. Jurkat cells expressing DC-SIGN or L-SIGN were clearly infected by Ebola virus Zaire and Reston GP-pseudotyped lentiviral vectors, indicating that expression of either of these two lectins in Jurkat cells is sufficient to confer permissivity (Fig. 2A). The DC-SIGN and L-SIGN dependency of the Jurkat cell infection was confirmed

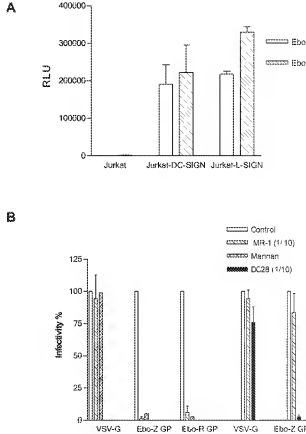

FIG. 2. (A) Jurkat cells expressing DC-SIGN and L-SIGN are permissive for Ebola virus infection. Control Jurkat cells or Jurkat cells expressing DC-SIGN or L-SIGN by transduction with a retroviral vector were infected with Ebola virus Zaire (Ebo-Z) or Reston (Ebo-R) GP-pseudotyped lentivirus (mean ± standard error, $n = 3$). (B) Specificity of DC-SIGN- and L-SIGN-mediated infectivity of Jurkat cells. DC-SIGN- and L-SIGN-mediated infectivity of Jurkat cells transduced with the retroviral vectors mentioned above was assessed by preincubation with mannan and specific monoclonal antibodies: MR-1 is DC-SIGN specific, and DC28 exhibits specificity for both DC-SIGN and L-SIGN. Results are shown as the percentage of luciferase activity compared to that of the untreated cells (mean ± standard error, $n = 3$). Mannan was tested once on Jurkat-DC-SIGN. DC28 was used only for Jurkat L-SIGN.

by the clear reduction of infectivity in the presence of mannan and anti-DC-SIGN and anti-L-SIGN antibodies, whereas a VSV-G-pseudotyped control was unaffected (Fig. 2B). Our results clearly indicate that DC-SIGN and L-SIGN are implicated in Ebola virus GP-mediated cell infection; however, the contribution and the specific molecular interactions of DC-SIGN and L-SIGN in Ebola virus cell entry remain to be defined. In this respect, and since many cells known to be susceptible to Ebola virus do not express these lectins, our results, like those recently reported for HIV and SIV (11), support the hypothesis that DC-SIGN and L-SIGN bind and concentrate Ebola virus to the cell membrane, thus facilitating the interaction in *cis* with cofactors required for cell entry, the low density of which may be limiting for infection of certain cell types.

Finally, the role of DC-SIGN–Ebola virus GP interaction on DC was explored by using monocyte-derived DC (MDDC). MDDC were obtained from blood monocytes according to a standardized protocol (14). Cells were cultured for 5 to 7 days in the presence of granulocyte-macrophage colony-stimulating factor and interleukin-4 to obtain a population of immature MDDC. DC were infected with the lentiviruses pseudotyped with VSV-G and Ebola virus GP (MOI of 10 and 0.1, respectively). Forty-eight to 72 h postinfection, cells were assayed for luciferase expression as described before. Infection of MDDC, although at a low level, was demonstrated by using a VSV-G-pseudotyped control. However, under the conditions used in our experiments and in spite of the high DC-SIGN expression of MDDC, we were unable to readily detect luciferase expression upon infection with Ebola virus GP-pseudotyped lentiviral vectors (data not shown). In this respect, and taking into account the evidence of Ebola virus infection of DC in vitro and in vivo (4), it is possible that limitations of the lentivirus pseudotyping approach, such as low titers or the requirement of additional viral products for entry into DC, might account for this negative result. We next tested whether DC-SIGN on the surface of DC could bind Ebola virus GP-pseudotyped viral particles and facilitate subsequent infection of susceptible cells (Fig. 3). DC were preincubated (150,000 cells in 100 μl) for 20 min at room temperature in the presence or absence of the DC-SIGN-specific antibody MR-1. Supernatants (300 μl) containing Ebola virus GP- or VSV-G-pseudotyped lentiviral particles were then added, and cells were maintained in rotation at room temperature for 2 h. Cells were washed four times in phosphate-buffered saline (PBS)–2% FBS, resuspended in 300 μl of fresh medium, and added to HeLa cells plated in 24-well plates. The same amount of supernatant maintained at room temperature without DC was used as control of infectivity. After 48 h of cocultivation, wells were washed twice with PBS, and HeLa cells were assayed for luciferase activity as described above. The infectivity achieved by cocultivation of HeLa and MDDC, incubated with a high-titer VSV-G-pseudotyped lentiviral supernatant and extensively washed, was more than 2 orders of magnitude lower than that of the initial non-cell-incubated supernatant. The remaining infectivity was unaffected by preincubation of MDDC with a DC-SIGN-specific antibody suggesting that it was most likely due to unspecific binding. In contrast, MDDC incubated with infectious supernatants of Ebola virus GP-pseudotyped viruses retained a higher proportion of the infectivity of the supernatant after

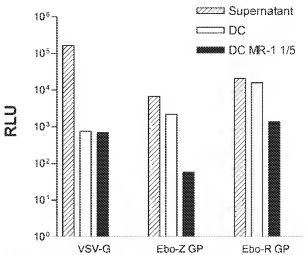

FIG. 3. MDDC bind Ebola virus GP-pseudotyped particles and transmit infectivity to susceptible cells. MDDC were incubated with infectious supernatants containing VSV-G-, Ebola virus Zaire (Ebo-Z)-, or Ebola virus Reston (Ebo-R) GP-pseudotyped lentiviruses after a brief preincubation in the absence or presence of MR-1 DC-SIGN-specific monoclonal antibody. Cells were extensively washed thereafter and plated onto HeLa cells. The same amount of infectious supernatant (Sup) without incubation with DC was directly added to the HeLa cells as a control of the original infectivity. Cells were assayed for luciferase 48 h after infection. The experiment was performed with cells from two independent donors, and a representative result is shown.

extensive washing. This effect was significantly reduced by preincubating MDDC with a DC-SIGN-specific antibody, indicating that MDDC are capable, through DC-SIGN interactions, of binding Ebola virus GP-pseudotyped viruses, maintaining infectivity, and achieving efficient infection in *trans* of susceptible cells in a way similar to that described for lentiviruses (8).

We have found that expression of DC-SIGN and its homologue L-SIGN enhances infectivity of Ebola virus-susceptible cells and is sufficient to confer permissivity for Ebola virus GP-mediated infection to a nonsusceptible cell line. Also, DC-SIGN on the surface of DC appears to act as a *trans* receptor capable of binding Ebola virus GP-pseudotyped viruses and efficiently transmitting the infection to susceptible cells. DC-SIGN and L-SIGN appear to be universal binding factors for primate lentiviruses. Our data indicate that these molecules have extended participation in other viral infections. The role of these C-type lectins in Ebola virus primary infection and dissemination deserves further investigation.

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Human Cytomegalovirus Binding to DC-SIGN Is Required for Dendritic Cell Infection and Target Cell *trans*-Infection

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Summary

Cytomegalovirus (CMV) infection is characterized by host immunosuppression and multiorganic involvement. CMV-infected dendritic cells (DC) were recently shown to display reduced immune functions, but their role in virus dissemination is not clear. In this report, we demonstrated that CMV could be captured by DC through binding on DC-SIGN and subsequently transmitted to permissive cells. Moreover, blocking DC-SIGN by specific antibodies inhibited DC infection by primary CMV isolates and expression of DC-SIGN or its homolog DC-SIGNR rendered susceptible cells permissive to CMV infection. We demonstrated that CMV envelope glycoprotein B is a viral ligand for DC-SIGN and DC-SIGNR. These results provide new insights into the molecular interactions contributing to cell infection by CMV and extend DC-SIGN implication in virus propagation.

Introduction

Human Cytomegalovirus (CMV) is a double-strand DNA virus belonging to the herpesviridae family and is a ubiquitous pathogen in humans. CMV interaction with its host is characterized by a primary infection followed by lifelong persistence in the host organism and viral reactivation episodes. CMV infection is asymptomatic in most immunocompetent individuals because of an efficient antiviral immune response. In contrast, CMV remains a major cause of morbidity and mortality in newborn and immunocompromised patients, namely in organ-transplanted recipients or AIDS patients. In any cases, CMV disease is characterized by a wide viral spread toward multiple organs (i.e., salivary glands, lung, kidney, gastrointestinal tract, liver, retina, CNS).

In vitro, a number of cell types are susceptible to CMV infection when considering virus entry and viral immediate early gene expression. However, full replication of virus DNA and subsequent progeny of infectious virions is limited to permissive cells (i.e., fibroblasts, endothelial cells, the U373 MG astrocytoma cell line, etc.; for review see Plachter et al., 1996). In fibroblasts (the prototypic cell type for in vitro studies of CMV infection), CMV entry occurs in sequential steps involving several viral envelope (Env) glycoproteins. Initial attachment of virus to host cells is mediated through interaction between Env glycoproteins gB (CMV gB) and/or CMV gM with cell surface heparan sulfate proteoglycans (Compton et al., 1993; Kari and Gehrz, 1992). Thereafter, binding of CMV gB with non-heparin cellular receptors probably allows more stable attachment of virus to cell surface (Boyle and Compton, 1998). Subsequent pH-independent fusion events between viral envelope and cell membrane are necessary for viral entry (Compton et al., 1992; Milne et al., 1998). Cell proteins involved in CMV attachment and/or fusion have not been identified precisely, although two candidates have been proposed. The first one is annexin II, which interacts with CMV gB (Pietropaolo and Compton, 1997). The second one is a 92.5 kDa protein binding to CMV gH (Baldwin et al., 2000). Fusion events are followed by penetration of the capsid which is transported to the nucleus. In some permissive cells, such as retinal pigment epithelial cells, CMV can also penetrate into cells by a mechanism of endocytosis (Bodaghi et al., 1999).

Recently, DC, which are refractory to infection by laboratory-adapted CMV strains, were shown to be permissive to CMV infection and replication when infected with primary, clinical viral isolates (Riegler et al., 2000). The mechanism of CMV entry into DC has not been investigated yet. It was recently shown that DC express a lectin called DC-SIGN (DC-Specific ICAM-Grabbing Nonintegrin). DC-SIGN, also called CD209, is a ligand for IntraCellular Adhesion Molecule-2 (ICAM-2) and ICAM-3 (Geijtenbeek et al., 2000a, 2000c) and is involved in the attachment of Human Immunodeficiency Virus-1 (HIV-1) (Geijtenbeek et al., 2000b) and Ebola (Alvarez et al., 2002) to DC. DC-SIGN was originally cloned from a placental cDNA library on the basis of its capacity to bind to the surface subunit HIV-1 Env glycoprotein 120 (HIV-1 gp120) (Curtis et al., 1992). DC-SIGN mediates HIV binding and internalization into DC, conferring to these cells the ability to transmit HIV to permissive $CD4^+$ T cells independently from HIV-1 replication (Geijtenbeek et al., 2000b). These findings suggest that DC-SIGN efficiently captures HIV-1 at mucosal sites of inoculation and facilitates its transport to sites of infection by using the migra-

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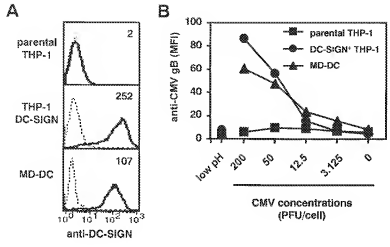

Figure 1. DC-SIGN-Expressing Cells Bind CMV on Their Surface

(A) Detection of DC-SIGN. Anti-DC-SIGN 1B10 mAb (bold line). Irrelevant isotypic control mAb (dotted line). Mean fluorescence intensity (MFI) values are indicated.

(B) Binding of CMV AD169 strain to cells expressing or not DC-SIGN was revealed by an anti-envelope glycoprotein B (CMV gB) mAb. Incubation in low pH buffer (200 PFU/cell) prior to staining abrogates detection of CMV gB.

tory ability of DC toward lymphoid organs (Banchereau and Steinman, 1998). A homolog of DC-SIGN, DC-SIGNR, was recently identified on the surface of endothelial cells and shown to display the same HIV-1 binding and *trans*-infection enhancement capacities shown by DC-SIGN (Bashirova et al., 2001; Pohlmann et al., 2001b). The putative role of DC-SIGN or DC-SIGNR in herpesvirus attachment to DC or endothelial cells has never been reported.

In the present study, we analyzed the mechanisms of CMV attachment to DC and the role of DC-SIGN in this process. We demonstrated that CMV is able to bind DC- and DC-SIGN-expressing THP-1 cells through direct interaction of DC-SIGN with viral envelope CMV gB. This binding leads to two independent results: (1) the transmission of DC-SIGN-bound infectious viral particles to different permissive cells and (2) an enhanced infection and CMV replication in DC- and DC-SIGN-expressing THP-1 cells.

Results

Expression of DC-SIGN at the Cell Membrane Enables Binding of CMV

We investigated first the capacity of CMV to bind DC-SIGN. Parental and DC-SIGN$^+$ THP-1 cells, or immature monocyte-derived DC (MD-DC) were incubated on ice with increasing concentrations of CMV, and the presence of cell-bound virions was quantified by flow cytometry using a mAb directed against the CMV gB. While parental THP-1 cells failed to bind detectable amounts of CMV, both DC-SIGN-expressing THP-1 and MD-DC absorbed CMV virions in a dose-dependent manner (Figures 1A and 1B). Prevention of CMV gB antibody-labeling by acidic washes proved the existence of cell-bound virions (Figure 1B). Abrogation of virion attachment observed following preincubation of cells with mannan, a complex sugar that binds to the Carbohydrate Recognition Domain (CRD) of lectins, suggests that the CMV-DC-SIGN interaction is accounted by the glycosylated residues of CMV envelope glycoproteins.

Transmission of CMV Infection to Permissive Cells Is Mediated by DC-SIGN

MD-DC, THP-1, or HeLa expressing DC-SIGN were incubated with a mutant CMV strain encoding a GFP (ADGFP) (Borst et al., 2001), washed, and cultured with permissive MRC5 cells. HeLa cells were selected for their refractoriness to CMV infection (Einhorn et al., 1982) which persists despite transduction with DC-SIGN (our unpublished data). MD-DC (Figure 2A), DC-SIGN$^+$ THP-1 (Figure 2B), and DC-SIGN$^+$ HeLa cells (Figure 2C), in contrast to parental THP-1 or HeLa cells, conveyed CMV infection as proved by the expression of GFP in MRC-5 cells. *Trans*-infection of MRC-5 cells was prevented by preincubating MD-DC, DC-SIGN$^+$ THP-1, or DC-SIGN$^+$ HeLa cells either with EGTA or mannan before being pulsed with CMV. Moreover, the anti-DC-SIGN mAb 1B10, which blocks HIV transmission (data not shown), also inhibited efficiently the transmission of CMV from DC-SIGN$^+$ cells to MRC-5 cells. We conclude that transmission of CMV to susceptible cells is accounted for by DC-SIGN and does not require productive infection by DC-SIGN-expressing cells.

The capacity of DC-SIGN to enhance infectiveness of CMV was assessed. To these purposes, MRC-5 cells were either incubated with low titers of cell-free CMV or cocultured with MD-DC (Figure 3A) or DC-SIGN$^+$ THP-1 (Figure 3B) previously pulsed with an identical amount of CMV. Coculture of MRC-5 with CMV-pulsed DC-SIGN$^+$ cells leads to a substantial enhancement of MRC-5 infections as compared to MRC-5 exposed to cell-free virus. The enhancement of CMV infectivity conferred by DC-SIGN$^+$ cells pulsed with CMV was abrogated by specific anti-DC-SIGN mAb 1B10 (Figures 3A and 3B). To determine if DC-SIGN-bound CMV retains infectivity over a more prolonged period of time than free virus, DC-SIGN$^+$ THP-1 were pulsed with CMV, washed, and cultured at 37°C for different periods before coculture with MRC-5 cells. In parallel, cell-free virus was incubated for the same period of time at 37°C before being added to MRC-5 cells. Our findings show that CMV remains infectious for 4–5 days when bound to DC-SIGN whereas cell-free virus retains its infectivity only for 2 days (Figure 3C).

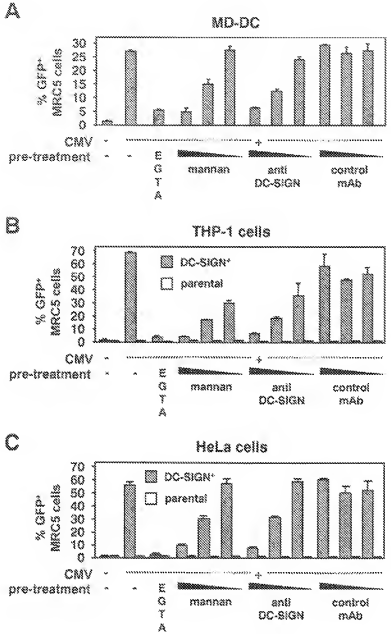

Figure 2. DC-SIGN-Dependent *trans*-Infection of CMV-Permissive Cells

(A) MD-DC, (B) DC-SIGN$^+$ versus parental THP-1 cells, or (C) parental versus DC-SIGN$^+$ HeLa cells were pretreated with EGTA (5 mM), mannan (20, 1, 0.05 μg/ml), anti-DC-SIGN 1B10 mAb (20, 1, 0.05 μg/ml), or an isotypic control (20, 1, 0.05 μg/ml) prior to incubation with ADGFP strain (1 PFU/cell). After removal of unbound virus and competitors, ADGFP-challenged cells were cocultured for 3 days with MRC-5 cells. Infection of MRC-5 was assessed by counting the number of GFP-expressing cells by flow cytometry.

In parallel, the detection by immunostaining of early markers of CMV replication (intranuclear immediate early and early antigens, IEA and EA, respectively) has been done (Figure 4A). The findings obtained by this alternative assay confirmed the role of DC-SIGN in the transmission of CMV to permissive cells and validated the *trans*-infection assay. Transmission of CMV from DC-SIGN$^+$ cells is not restricted to a particular permissive cell type since DC-SIGN$^+$ THP-1 cells also transmitted infectious virions to the U373 MG astrocytoma cell line (Figure 4B).

We next aimed at determining if other members of the herpesviridae family have the same capacity as CMV to interact with DC-SIGN. To this purpose, DC-SIGN$^+$ THP-1 cells were exposed to clinical isolate of CMV, HSV-1, or VZV and thereafter cocultured with MRC-5 cells which are fully susceptible to the three viruses (Figure 4D). Expression of CMV- but not HSV-1- or VZV-EA or -IEA in MRC-5 cells is compatible with a high degree of specificity for the interaction of DC-SIGN with CMV envelope glycoproteins (Figure 4C).

DC-SIGN Cytoplasmic Tail Is Critical for Enhanced Transmission of CMV

The role proposed for DC-SIGN internalization for *trans*-enhancement of HIV infection was assessed for CMV transmission from DC-SIGN$^+$ cells to susceptible cells. To this purpose, THP-1 cells expressing mutant forms of DC-SIGN (Kwon et al., 2002) encoding either combined deletion of dileucine- and tyrosine-based motifs (DC-

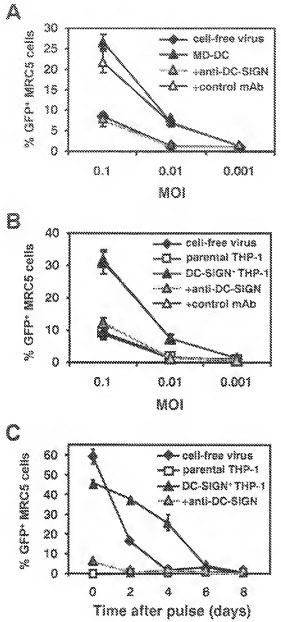

Figure 3. DC-SIGN Enhances CMV *trans*-Infection of MRC-5 Cells and Retains Long-Term Infectious Virus

(A) MD-DC, (B) parental, or DC-SIGN$^+$ THP-1 cells were incubated with ADGFP in the absence or in the presence either of anti-DC-SIGN 1B10 mAb or an isotypic, control mAb. Thereafter, cells were cocultured with reporter MRC-5 cells.

(C) DC-SIGN$^+$ and parental THP-1 cells were incubated (4 hr) with ADGFP (moi ~ 1) and washed thereafter. At days 0, 2, 4, 6, and 8 after pulse, ADGFP-challenged cells were cocultured with reporter MRC-5 cells for 3 days. In (A), (B), and (C), MRC-5 cells were incubated with the corresponding amount of cell-free virus to monitor kinetic and extent of infection. Values represent the percentage of MRC-5 cells expressing GFP.

SIGN Δ35), or the dileucine-based motif only (DC-SIGN Δ20), which are putative internalization motifs required for DC-SIGN endocytosis, were exposed to low MOI CMV infection. Both DC-SIGN mutants were expressed in THP-1 cells with similar efficiency as the wild-type counterpart (Figure 5A). Moreover, they displayed roughly comparable capacities to bind CMV particles (Figure 5B). Parental and DC-SIGN-expressing (either wt or mutated) THP-1 cells were then assessed for their ability to transmit CMV to permissive MRC-5 cells. We found that following incubation with ADGFP CMV at 37°C, DC-SIGN Δ35- or DC-SIGN Δ20-expressing THP-1 cells showed a marked decreased capacity to transmit CMV as compared to DC-SIGN$^+$ THP-1 cells (Figure 5C). Incubation on ice of DC-SIGN wt-expressing THP-1 cells with CMV prevented virus transmission to MRC-5 cells (Figure 5C). These results suggest that, similarly to HIV infection, *trans*-enhancement of CMV infection by DC-SIGN-expressing cells requires the cytoplasmic domain of DC-SIGN.

DC-SIGN Expression Renders Low-Susceptible Cells Sensitive to CMV Infection and Mediates the Infection of MD-DC by Primary CMV Isolates

We next investigated whether DC-SIGN is involved in *cis* in the entry of CMV into host cells. Two complementary approaches were developed to this purpose. First, using either HEK 293T or THP-1 cells transduced with DC-SIGN, we evaluated their capacity to support CMV infection. It has been previously reported that undifferentiated THP-1 are unable to support CMV IE gene expression despite virus entry (Lashmit et al., 1998; Weinshenker et al., 1988). We confirmed this finding and show that the HEK 293T cell line similarly appears to be poorly susceptible to CMV infection (ADGFP virus). In sharp contrast with these findings, both HEK 293T and THP-1 expressing DC-SIGN were highly susceptible to CMV infection. Indeed, more than 40% of DC-SIGN$^+$ THP-1 cells were positive for GFP after 2 hr of contact with CMV ADGFP followed by a 2 day incubation, while no GFP expression was found in parental cells (Figure 6A). Similarly to DC-SIGN, the homologous DC-SIGNR lectin was capable of rendering HEK 293T susceptible to CMV infection (Figure 6B). Conclusive evidence about the role played by DC-SIGN in the infectiveness of transduced cells came from the drastic reduction of the CMV IE gene expression levels in both DC-SIGN$^+$ THP-1 and HEK 293T cells in the presence of anti-DC-SIGN mAb (Figure 6A).

MD-DC, which show natural expression of DC-SIGN, were used to confirm and extend the findings observed in the first set of experiments. By opposition to THP-1 cells, MD-DC are known to be permissive to infection by primary CMV isolates. Detection of IEA and EA in a substantial number of MD-DC when incubated with TB40/E proved the susceptibility of these cells to non-adapted, clinical CMV strains. Amazingly, preincubation of MD-DC with the anti-DC-SIGN 1B10 mAb prevented their infection by CMV with roughly the same efficiency as it did in DC-SIGN$^+$ THP-1 cells (Figure 6C).

Full replication of CMV in DC-SIGN-expressing cells was then assessed by quantifying the progeny of infectious virions. MRC-5, MD-DC, DC-SIGN$^+$, or parental THP-1 cells were incubated with low titers of a primary CMV strain, washed in acidic buffer to remove noninternalized virus, and thereafter cultured for 14 days. The generation of infectious CMV virions from these cells

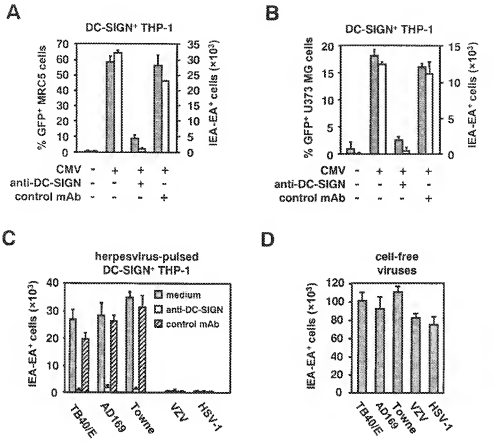

Figure 4. DC-SIGN Mediates CMV Transmission to Different Permissive Cells but Does Not Allow Transmission of HSV-1 and VZV

(A and B) DC-SIGN+ THP-1 cells were exposed either to anti-DC-SIGN 1B10 mAb or an isotypic control mAb prior to infection with ADGFP. ADGFP-pulsed DC-SIGN+ THP-1 cells were cocultured for 24 hr either with MRC-5 (A) or U373 MG cells (B). GFP- (gray bars) or CMV IEA/EA-expressing cells (open bars) were counted to evaluate CMV infection.

(C) DC-SIGN+ THP-1 cells incubated with anti-DC-SIGN 1B10 mAb (open bars) or an isotypic control mAb (dashed bars) or left untreated (gray bars) were exposed for 1 hr to TB40/E, AD169, or Towne CMV isolates, HSV-1, or VZV. After removal of unbound virus and mAb, cells were cocultured with MRC-5 reporter cell line for 5 days.

(D) MRC-5 cells were exposed for 5 days to an identical amount of cell-free viruses as used in (C) to infect DC-SIGN+ THP-1 cells. IEA/EA-expressing cells were detected by immunocytochemistry using specific antibodies for each herpesvirus.

was quantified by plaque assay titration on MRC-5 cells. Accumulation of CMV virions was detected in culture supernatants from MD-DC and DC-SIGN+ THP-1 cells (Figure 6D). The amount of infectious virions released by MD-DC- or DC-SIGN-expressing THP-1 were 10 and 16 times, respectively, more elevated than the number of input virus used at day 0 and comparable to amounts released by MRC-5 cells (Figure 6D). Preincubation of MD-DC or DC-SIGN+ THP-1 with the specific anti-DC-SIGN 1B10 mAb precluded detectable generation of CMV infectious virions, thus demonstrating the involvement of DC-SIGN in the *cis*-infection of DC-SIGN-expressing cells (Figure 6D).

Hence, these results imply that in *cis* cell surface expression of DC-SIGN not only potentiates the expression of CMV IE gene products but also confers to CMV low-susceptible cells the capacity to support a full replicative cycle in the host cell. These findings suggest a crucial biological role of DC-SIGN in the propagation of the CMV natural infection by DC.

Identification of CMV Glycoprotein B as a Viral Ligand of DC-SIGN

Since DC-SIGN was shown to bind HIV particles through a specific interaction between the carbohydrate recognition domain (CRD) of DC-SIGN and sugar moities of HIV-1 gp120 (Mitchell et al., 2001), we searched for an equivalent of HIV-1 gp120 on CMV particles. The human CMV virion is known to harbor several different envelope glycoproteins. Among them, CMV gB, gH, and gM were shown to be directly involved in two early events of the CMV infection: CMV attachment and fusion between viral and cellular membranes (Compton et al., 1993; Kari and Gehrz, 1992; Milne et al., 1998). The reasons for focusing our research on CMV gB are manifold. First, CMV gB is the most abundant and the most extensively N- and O-glycosylated envelope glycoprotein of CMV (Gibson, 1983). Second, it has been demonstrated that sequence variations in CMV gB from different strains of human CMV are lower than in other CMV envelope glycoproteins (Chou and Dennison, 1991). Third, CMV

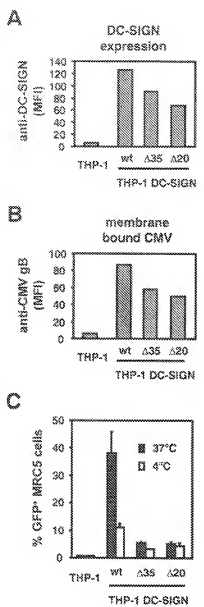

Figure 5. Requirement of DC-SIGN Cytoplasmic Domain for CMV Transmission

(A) Cell surface expression of wt or mutated DC-SIGN (Δ35 and Δ20) receptors analyzed by immunostaining (1B10 mAb) and flow cytometry.

(B) CMV binding capacity of THP-1 expressing wt or truncated DC-SIGN revealed by anti-CMV gB mAb.

(C) Parental or DC-SIGN$^+$ cells were incubated, either at 4°C or at 37°C for 2 hr, with ADGFP CMV (moi = 0.1) and cocultured with MRC-5 cells for 3 days. Infection was assessed by estimating the number of GFP-expressing cells.

gB has been proposed to play central roles in virion penetration into cells, transmission from cell to cell, and fusion of infected cells (Navarro et al., 1993).

Recombinant, biotinylated CMV gB was directly bound and detected on DC-SIGN-expressing THP-1 cells or MD-DC, but not on parental THP-1 (Figure 7A), and similar findings were observed with unlabeled CMV gB (data not shown). The attachment of CMV gB to cells was specifically abrogated by preincubation with the blocking anti-DC-SIGN 1B10 mAb. Further authentication of CMV gB as a CMV DC-SIGN ligand came from a competition assay with other viral envelope glycoproteins. In this assay, we preincubated DC-SIGN$^+$ THP-1 cells with purified HIV-1 gp120, CMV gB, HSV-1 gB, VZV gB, HSV-1 gD, or VZV gE. Following exposure to each single envelope glycoprotein, cells were incubated with biotinylated HIV-1 gp120, the binding of which to DC-SIGN$^+$ THP-1 cells was evidenced by immunostaining and FACS analysis. Among the herpesvirus proteins assessed, only CMV gB decreased the binding of biotinylated HIV-1 gp120 on DC-SIGN. This competitive effect of CMV gB was almost as efficient as that shown by unlabeled HIV-1 gp120, mannan, or anti-DC-SIGN mAb 1B10 (Figure 7B). Pretreatment of DC-SIGN$^+$ THP-1 cells and MD-DC with recombinant CMV gB before incubation with CMV virions also efficiently blocked transmission of CMV to susceptible MRC-5 cells (data not shown).

To investigate whether DC-SIGNR could also bind to CMV gB, we incubated HEK 293T cells transiently transfected with cDNA encoding DC-SIGN or DC-SIGNR in the presence of biotinylated-HIV-1 gp120, -CMV gB, or -BSA (Figure 7C). No binding was observed when incubating transfected cells with the control BSA. In contrast, both HIV-1 gp120 and CMV gB efficiently bound to HEK 293T cells expressing either DC-SIGN or DC-SIGNR. Both interactions were calcium dependent since they were blocked by EGTA (data not shown). Surprisingly, at low concentrations CMV gB displayed a higher apparent affinity than HIV-1 gp120 for DC-SIGNR, whereas both viral glycoproteins bound to DC-SIGN-expressing cells with comparable efficiency. Together, these results demonstrated that CMV gB is a CMV ligand for DC-SIGN and DC-SIGNR. It deserves to be investigated whether this capacity is restricted to CMV gB or is shared by other CMV envelope glycoproteins.

Characterization of DC-SIGN-Glycoprotein Interactions

The surface plasmon resonance (SPR) technology was used to further analyze the characteristics of DC-SIGN binding to HIV-1 gp120 and CMV gB in vitro. Typical sensorgrams were obtained by injection of a concentration range of recombinant soluble CRD domain of DC-SIGN (0.13 to 1 μM) over surfaces functionalized with HIV-1 gp120 (Figure 7D, left panel), CMV gB (Figure 7D, middle panel), or HSV-1 gB (Figure 7D, right panel). Visual inspection of the binding curves immediately showed that DC-SIGN binds to HIV-1 gp120 and CMV gB, while only displaying negligible binding to HSV-1 gB. Binding of DC-SIGN CRD to both HIV-1 gp120 and CMV gB was strongly inhibited by the anti-DC-SIGN 1B10 mAb and EDTA (data not shown). The binding curves were then individually fitted to a Langmuir model (A + B ⇌ AB). This analysis returned an average on rate $k_{on} = 3.33 \times 10^3\ M^{-1}S^{-1}$, and off rate $k_{off} = 1.01 \times 10^{-3}\ S^{-1}$, thus giving an equilibrium dissociation constant of 0.30 μM for HIV-1 gp120, and $k_{on} = 4.4 \times 10^5\ M^{-1}S^{-1}$, $k_{off} = 1.26 \times 10^{-3}\ S^{-1}$, leading to an equilibrium dissociation constant of 0.29 μM for CMV gB. Since the affinities that characterize the DC-SIGN CRD binding to HIV-1 gp120 and to CMV gB are similar, the higher binding

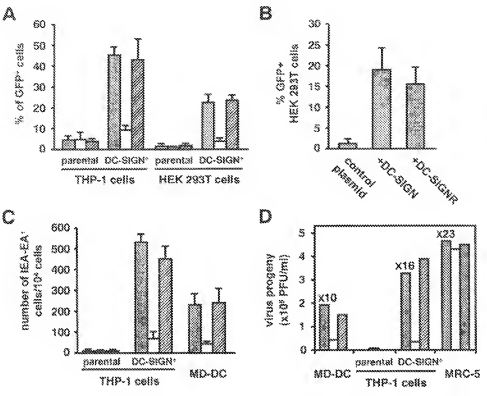

Figure 6. DC-SIGN Expression Renders Susceptible Cells Permissive to CMV Infection

(A) Cells were pretreated for 30 min with medium (gray bars), anti-DC-SIGN 1B10 mAb (open bars), or an isotypic control mAb (dashed bars) and thereafter incubated with ADGFP strain (1 PFU/cell). HEK 293T were transiently transfected either with a DC-SIGN cDNA plasmid or a control plasmid (pcDNA3.1).

(B) HEK 293T cells were transiently transduced with DC-SIGN or DC-SIGNR cDNAs and incubated with ADGFP. In (A) and (B), GFP-expressing cells were quantified by flow cytometry at 3 days after infection.

(C) THP-1, DC-SIGN$^+$ THP-1 cells, or MD-DC were pretreated as described in (A) (same symbols) and infected with TB40/E CMV (moi = 1) for 3 days. CMV infection was assessed by immunostaining with specific CMV IEA/EA mAb. Total number of CMV IEA/EA-positive cells on the slide was determined by extrapolating the number of positive cells contained in the optical field of the microscope (×10 objective).

(D) MD-DC, MRC-5, parental, and DC-SIGN$^+$ THP-1 cells were pretreated and infected as described in (C). Noninternalized viral particles were removed by short incubation in a low pH buffer. At day 14 after infection, virions released in culture supernatants were titrated on MRC-5 cells by plaque assays. Numbers on top of histograms indicate the rate of viral amplification. These values were calculated by dividing the absolute number of CMV particles collected in supernatants by the absolute number of CMV particles used to infect cells (20,000 PFU).

level observed with the HIV-1 gp120 activated surface compared to the CMV gB surface (Figure 7D, left and middle panels) may simply reflect a difference in immobilization or in glycan density between both proteins.

Discussion

In the present report, we provide insights into the mechanisms of interaction of CMV with DC and the transmission of CMV infection to other cell targets. We show that DC-SIGN accounts for most of the binding of CMV to DC and mediates the attachment of CMV virions when expressed in DC-SIGN negative cells. Interaction of CMV with DC-SIGN occurs through specific binding with at least one CMV envelope glycoprotein, CMV gB. DC-SIGN is a type II membrane protein in which the extracellular domain encompasses the CRD and a stalk that mediates tetramerization (Mitchell et al., 2001). Like DC-SIGN, CMV gB is also present in multimeric complexes in CMV envelope (Scheffczik et al., 2001). CMV gB-DC-SIGN interactions analyzed by SPR were conducted using recombinant soluble forms of DC-SIGN CRD, which are monomers (our unpublished data). The affinity of CMV gB for DC-SIGN measured by SPR was 0.3 μM and was comparable to that estimated for HIV-1 gp120 (Mitchell et al., 2001). This relatively low affinity is likely due to the inability of CRD to multimerize. The estimated affinity (Kd) of HIV-1 gp120 for the natural DC-SIGN molecule is 1.4 nM (versus 5 nM for CD4) (Curtis et al., 1992). These findings suggest that like HIV-1 gp120, CMV gB would display high affinity for oligomerized DC-SIGN.

DC-SIGN-bound CMV retains infectious capacity since, upon binding onto DC-SIGN$^+$ THP-1 cells or MD-DC, CMV is transmitted to permissive cells where the virus replicates actively. DC are receptive to CMV infection by primary, nonadapted CMV isolates and refractory to infection by adapted, CMV laboratory strains. Taking advantage of this characteristic, we show that the capacity of DC to transmit CMV to permissive cell targets can be dissociated from the ability of CMV to infect and replicate in DC. This result was confirmed using DC-

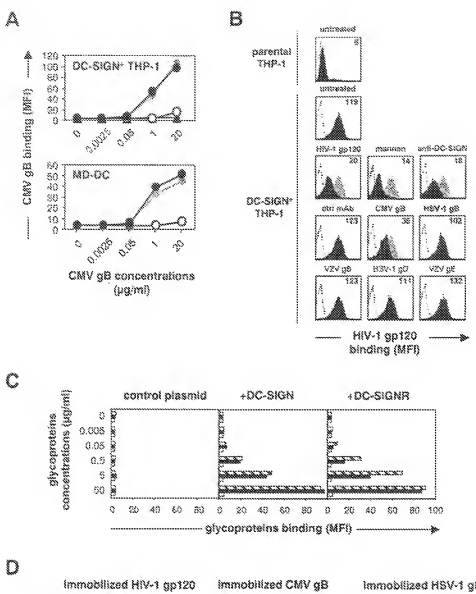

Figure 7. Identification of the CMV gB as a Viral Ligand for DC-SIGN and Characterization of the DC-SIGN/CMV gB Interaction by SPR

(A) Binding of CMV gB to DC-SIGN. DC-SIGN$^+$ THP-1 cells or MD-DC were pretreated with medium (black closed circles), anti-DC-SIGN 1B10 mAb (open circles), or an isotypic, control mAb (gray closed circles) and thereafter incubated with biotinylated CMV gB. Cell-bound CMV gB was revealed by PE-labeled streptavidin. Incubation of parental THP-1 cells with biotinylated CMV gB is also shown (black closed triangles).

(B) Competition assay of biotinylated HIV-1 gp120 binding to DC-SIGN. Parental (upper panel) or DC-SIGN$^+$ THP-1 cells (all other panels) were incubated with 2 μg/ml of biotinylated HIV-1 gp120. DC-SIGN$^+$ THP-1 cells were left untreated or preincubated with potential competitors (unlabeled HIV-1 gp120, mannan, anti-DC-SIGN 1B10 mAb, control isotypic mAb, or envelope glycoproteins from CMV gB, HSV-1 gB, and gD or VZV gE and gB) before incubation with biotinylated HIV-1 gp120. MFI of biotinylated HIV-1 gp120 staining is indicated in the upper-right corner of histograms. In each panel, control staining (dotted line) and biotinylated HIV-1 gp120 labeling in the absence of competitor (gray filled profile) or after preincubation with competitors (black filled histogram) are shown.

(C) Binding of CMV gB to DC-SIGNR. HEK 293T cells were transiently transfected either with a control plasmid or plasmids encoding DC-SIGN or DC-SIGNR cDNAs. Transfected cells were incubated with increasing concentrations of biotinylated-CMV gB (dashed bars), biotinylated-

SIGN$^+$ HeLa cells which were capable of transmitting CMV to permissive target cells, while CMV IE antigen expression was never detected in these refractory cells. This DC-SIGN function is reminiscent of the aptitude for transmission of infection to CD4$^+$ T lymphocytes shown either by HIV-1-pulsed-DC or -DC-SIGN-transduced cells.

Binding to and transfer of HIV-1 from DC-SIGN$^+$ cells appear to be separable steps (Pohlmann et al., 2001a). Recently, it has been shown that efficient transmission of HIV to CD4$^+$ T lymphocytes from DC-SIGN-expressing THP-1 cells requires internalization signals encoded in the cytoplasmic domain of the lectin (Kwon et al., 2002). The requirement of DC-SIGN cytoplasmic signals for efficient *trans*-infection (named *trans*-enhancement) becomes particularly evident when low amount of virus are used as inoculum (Geijtenbeek et al., 2000b). Similarly to HIV, suboptimal inoculums of CMV become highly infectious when transferred from DC-SIGN$^+$ THP-1 cells. Moreover, DC-SIGN derivatives Δ35 or Δ20 failed to support CMV transmission to highly susceptible cells, and incubation of CMV with wild-type- or truncated-DC-SIGN-expressing cells at 4°C prevented CMV transmission to permissive cells. These findings suggest that endocytosis of the receptor is required for efficient transmission of CMV to permissive cells. However, our experiments do not permit ruling out the involvement of putative transduction of intracellular signals in this phenomenon since deletion of DC-SIGN cytoplasmic domains or inhibition of cell signal activation at 4°C may preclude DC-SIGN-dependent cell activation. Overall, these findings suggest that in the natural CMV infection, DC-SIGN promotes take up of CMV and permits enhancement of CMV transmission by interstitial DC to other cells. The hypothesis of in vivo CMV transport by DC raised the question of the stability of DC-SIGN-bound CMV particles. As previously described for HIV, we evidenced that DC-SIGN$^+$ THP-1 cells can transmit CMV to other cell targets after 5 days in culture whereas cell-free virus lose infectivity upon incubation at 37°C for 24 to 48 hr. The ability of DC to transmit infection for a long time after exposure supports the hypothesis that DC transport small amounts of CMV from entry sites to target organs where they could transmit infectious CMV particles by cell-to-cell contact.

A striking feature of DC-SIGN-CMV interactions is the capacity of the lectin to facilitate the infection of low-susceptible cells by CMV. Thus, THP-1 cells that do not normally support CMV replication become productively infected as they express DC-SIGN. CMV attachment to host cells is supposed to occur namely through low-affinity interactions with heparan sulfate proteoglycans (Compton et al., 1993; Kari and Gehrz, 1992). However, beyond this primary site of binding, the existence of an alternative cellular cofactor, required for a strong attachment of CMV on cell membranes as well as for its entry into cells, is postulated (Boyle and Compton, 1998). Annexin II, which binds to CMV gB (Pietropaolo and Compton, 1997) and a 92.5 kDa protein which binds to CMV gH (Baldwin et al., 2000) have been proposed to play this role. It is unlikely that DC-SIGN is the elusive CMV receptor that ultimately determines entry of the enveloped virions and replication in CMV infection-susceptible cells. Indeed, CMV entry and infection occur in a number of cell types (i.e., MRC-5 fibroblasts and U373 MG astrocytoma cells) where DC-SIGN is not expressed. The putative CMV receptor in these cells might be different from a lectin, although the existence of yet unidentified DC-SIGN-like molecules accounting for binding and entry of CMV cannot be formally ruled out.

The capacity of DC-SIGN to promote in *cis* CMV replication in otherwise low-susceptible cells can be explained by three not mutually exclusive hypotheses. DC-SIGN owns the capacity to capture and internalize HIV-1 in DC (Kwon et al., 2002). By analogy, DC-SIGN might promote internalization and trafficking of CMV to an intracellular compartment where it could initiate the infectious cycle. Alternatively, attachment of CMV to DC-SIGN, or DC-SIGNR, might facilitate the interaction with the authentic cellular receptor, which ultimately would account for CMV entry. Such a function would be reminiscent of the facilitating effect shown by DC-SIGN on HIV infection of T lymphocytes displaying low levels of CCR5 (Lee et al., 2001). Finally, differentiation of THP-1 cells with TPA was shown to induce permissiveness to CMV replication (Weinshenker et al., 1988). Similarly, signal transduction through DC-SIGN could lead to cellular differentiation and subsequent CMV replication.

Regarding CMV infection, the in *cis* capacity of DC-SIGN to facilitate viral entry is likely of biological relevance since the blockade by specific anti-DC-SIGN antibodies drastically reduces infectiveness of DC by primary, CMV isolates. The capacity of DC to support CMV infection may be related to the amount of DC-SIGN expressed at their surface. Thus, immature DC which express high levels of DC-SIGN can be infected by CMV (Raftery et al., 2001; Riegler et al., 2000), while matured DC that display low DC-SIGN expression show reduced susceptibility to CMV (our unpublished data). Expression of DC-SIGN on immature DC of intestinal and genital mucosae (Geijtenbeek et al., 2000b; Jameson et al., 2002) may confer to this cofactor a crucial role for the infection of these primary target cells at the anatomical sites where initial CMV transmission or propagation most probably take place. A recent study described a monocyte-derived macrophage circulating subset, expressing DC markers in vivo (Soderberg-Naucler et al., 1997). This subset was shown to harbor latent CMV which reactivates upon allogeneic stimulation. It appears necessary to investigate the expression of DC-

HIV-1 gp120 (black bars), or biotinylated-BSA (open bars). Binding of biotinylated proteins was revealed by PE-conjugated streptavidin and analyzed by flow cytometry. Values are represented as MFI.

(D) SPR analysis of DC-SIGN/CMV gB interaction. The recombinant soluble CRD of DC-SIGN at (from bottom to top) 0.13, 0.21, 0.36, 0.6, or 1 μM was injected over surfaces coated with HIV-1 gp120 (left panel), CMV gB (middle panel), or HSV-1 gB (right panel) to analyze the association phase, after which running, buffer alone was injected to analyze the dissociation phase. Binding responses (response unit, RU) are reported as a function of time. Dissociation constants (Kd) are indicated for left and middle panels.

SIGN by these cells which could represent a biological link between this newly identified dendritic-like subset and the results we provide in this report. As recently reported, CMV-infected DC display decreased antigen presentation and differentiation capacities (Andrews et al., 2001; Raftery et al., 2001). Hence, by promoting DC-mediated *trans*-infection of target cells as well as *cis*-infection of DC, DC-SIGN could be involved, apart from virus propagation, in CMV-mediated altered immune response.

We show that DC-SIGNR is also able to bind CMV gB and to promote *cis*-infection of apparently low-susceptible cells. This DC-SIGN homolog is mainly expressed on EC (Bashirova et al., 2001; Pohlmann et al., 2001b) which are known to be preferential targets of CMV in vivo and replicate primary, nonadapted CMV strains in vitro (Kahl et al., 2000). The expression of DC-SIGNR on placental EC and macrophages (Soilleux et al., 2001) could be involved in the materno-fetal transmission of CMV during congenital infections. Similarly, DC-SIGNR expressed in liver EC may be implicated in CMV-induced hepatitis, one of the most frequent clinical forms of this infection.

Murine CMV shares many essential characteristics with its human counterpart and has been a widely studied model for CMV infection. It has been shown that infection of DC by murine CMV prevents delivery of the signals required for T cell activation. The impairment of DC functions by murine CMV is supposed to be detrimental for the host immune responses (Andrews et al., 2001). The cloning of several homologs of DC-SIGN in mice (Park et al., 2001) should provide this model with an invaluable tool for studying the implication of DC-SIGN-like molecules in the dynamic of CMV dissemination, the role of the different subsets of DC in the course of CMV propagation, and eventually the causes of CMV-induced immunosuppression.

We hope that our findings will contribute to the definition of future strategies for prophylaxis of CMV infection. Thus, it could be envisaged that in vivo inhibition of CMV/DC-SIGN or DC-SIGNR interaction might result in reduced virus dissemination in the host organism through inhibition of DC-mediated *trans*-infection of in vivo susceptible cells and/or infection of DC-SIGN- or DC-SIGNR-expressing cells, namely EC or DC. Preventing DC infection should in turn protect antiviral immune response by blocking the suppressive effect of CMV on these cells.

Experimental Procedures

Herpesviruses

AD169, Towne (CMV laboratory strains), and TB40/E (CMV clinical isolate) were provided by Dr. S. Michelson (Institut Pasteur, Paris) and Dr. C. Sinzger (Tubingen, Germany), respectively. ADGFP is a genetically modified AD169 strain encoding an Enhanced Green Fluorescent Protein driven by the CMV immediate-early gene promoter (Borst et al., 2001). VZV and HSV-1 clinical isolates were obtained from Dr. Isabelle Garrigue (Laboratory of Virology, CHU Pellegrin, Bordeaux, France).

Reagents, Antibodies, and Viral Glycoproteins

Mannan and EGTA were purchased from Sigma-Aldrich Corporation (Saint Louis, MO). Soluble viral envelope glycoproteins were produced and purified from mammalian or insect cells. HIV-1 gp120 (MN isolate) was obtained from the NIBSC repository (Medical Research Council, United Kingdom). VZV gB and VZV gE (Jacquet et al., 1995) were gifts from Dr. A. Jacquet (Department of Applied Genetics, Gosselies, Belgium). HSV-1 gB and HSV-1 gD (Sisk et al., 1994) were provided by Dr. G.H. Cohen (University of Pennsylvania, Philadelphia). Expression and purification of CMV gB (gift of Dr. Claude Meric, Aventis Pasteur, Marcy l'Etoile, France) were previously described (Norais et al., 1996; Pass et al., 1999). Anti-CMV gB (clone 1-M-12, IgG1) and anti-DC-SIGNR (clone 120604, IgG2a) mAbs were purchased from Biodesign International (Saco, ME) and R&D Systems (Minneapolis, MN), respectively. Anti-LIF 7D2 (Taupin et al., 1993) and anti-SDF-1 K15C monoclonal antibodies (mAb) (Amara et al., 1999) were used as isotypic controls.

Cells

MRC-5 (Bio Mérieux S.A., Marcy l'Etoile, France) and U373 MG (ECCC, Salisbury, United Kingdom) are CMV-, HSV-1- and VZV-permissive cell lines, from fibroblastic and astrocytic origin, respectively. Parental and DC-SIGN$^+$ THP-1 cells (wild-type and Δ35 and Δ20 mutants lacking the first 35 and 20 amino acids of the cytoplasmic domain, respectively) (Kwon et al., 2002) were a gift from Dr. D.R. Littmann (Skirball Institute of Biomolecular Medicine, New York). DC-SIGN$^+$ HeLa cells were generated by infecting HeLa cells with an HIV-derived vector (TRIP-ΔU3 vector, a gift from Dr. P. Charneau, Institut Pasteur, Paris) encoding a human DC-SIGN cDNA. MD-DC were generated from peripheral blood monocytes treated with 20 ng/ml IL-4 (Schering-Plough, Kenilworth, NJ) and 100 ng/ml GM-CSF (Leucomax, Novartis-Pharma, Rueil Malmaison, France) (Romani et al., 1994). At day 5, virtually the totality of cells displayed the phenotype CD1a$^+$, HLA-DR$^+$, CD80^{low}, CD86^{low}, CD83$^-$, CD14$^-$ characteristic of immature MD-DC.

DC-SIGN cDNA and anti-DC-SIGN Antibodies

DC-SIGN cDNA was isolated from human immature MD-DC by RT-PCR. For expression in mammalian cells, human DC-SIGN was subcloned at the EcoRI/XbaI sites of the pcDNA3 myc-His (version A) plasmid (Invitrogen, Carlsbad, CA). The DC-SIGNR cDNA was a gift from Dr. R.W. Doms (University of Pennsylvania, Philadelphia). Anti-DC-SIGN clone 1B10 (IgG2a) was obtained by immunizing BALB/c mice with HEK 293T cells transfected with DC-SIGN cDNA, screened by indirect immunofluorescent staining and FACS analysis on DC-SIGN$^+$ HeLa cells and used as purified immunoglobulins.

Infection Assays

For *trans*-infection experiments, cells were incubated with viral suspensions (CMV, VZV, or HSV-1, moi = 1) for 2 hr at 37°C. Thereafter, unbound viral particles were removed by extensive washes and cells were cocultured with subconfluent MRC-5 or U373 MG cell monolayers. After 24 to 72 hr, infected MRC-5 or U373-MG cells were fixed, permeabilized, and stained with specific mAbs directed against IEA- or EA-CMV (mAbs E13 and 2A2, respectively), VZV (mAb 2013), or HSV (mAb CHA-437) (Argen Biosoft, Varilhes, France). When indicated, MD-DC or THP-1 (parental or DC-SIGN$^-$) cells were incubated with EGTA (5 mM), mannan, or anti-DC-SIGN (1B10 mAb) for 30 min at 4°C prior to challenge with infectious preparations. Infection by ADGFP strain was assessed by counting GFP-expressing cells at day 3 by flow cytometry. For long-term infectivity experiments, DC-SIGN$^+$ or parental THP-1 cells were incubated with ADGFP (moi = 1) for 4 hr at 37°C. After extensive washes, infected cells were incubated at 37°C, and an aliquot of these cells was added to a subconfluent MRC-5 cell culture every 2 days during the assay.

To assess the effect in *cis* of DC-SIGN during infection, cells were incubated with low titers of CMV (moi = 0.1) for 2 hr at 37°C. Noninternalized viral particles were removed by washes in low pH citrate buffer (pH = 3). The number of infected cells was determined by immunocytochemistry 72 hr after infection. Supernatants from infected cells kept in culture for 14 days were harvested to quantify de novo generated virions by plaque-assay titration.

HIV-1 gp120 Binding Competition and CMV gB Direct Binding Assays

DC-SIGN$^+$ THP-1 cells were washed two times, resuspended in ice-cold binding buffer (1 mM $CaCl_2$, 2 mM $MgCl_2$, and 0,1% Bovine

Serum Albumin in PBS) at 10^6 cells/ml and pretreated or not for 15 min with competitors (20 μg/ml). Thereafter, recombinant biotinylated CXCR4-tropic (MN isolate) HIV-1 gp120 (2 μg/ml; Immunodiagnostics Inc., Woburn, MA) was added for 30 min at 4°C. After extensive washing, cell-bound biotinylated HIV-1 gp120 was revealed by flow cytometry using FITC-conjugated Streptavidin (Immunotech SA, Marseille, France). For CMV gB binding experiments, recombinant soluble CMV gB and Bovine Serum Albumine (BSA; Amersham Pharmacia Biotech, Uppsala, Sweden) were biotinylated with sulfo-NHS biotin, according to manufacturer instructions (Pierce, Rockford, IL).

Analysis of DC-SIGN Interactions with Viral Envelope Glycoproteins by SPR

The cDNA coding for the DC-SIGN CRD (amino acids 254–404) was obtained by PCR and cloned into pET15b (Novagen). The protein was expressed in *Escherichia coli* C41(DE3) as inclusion bodies. Refolding of the protein has been done by dilution and dialysis as described (Mitchell et al., 2001). Purification of refolded DC-SIGN CRD has been achieved in two steps: first on a Ni-NTA (QIAGEN) column equilibrated in 25 mM Tris Cl (pH 7.8), 150 mM NaCl, and 4 mM $CaCl_2$ (loading buffer) and eluted with a linear gradient of imidazole and second on a Mannose-agarose column equilibrated in loading buffer, and eluted in buffer where $CaCl_2$ was replaced by EDTA (10 mM). Pooled fractions are then concentrated and dialyzed against loading buffer.

Four flow cells of a Biacore B1 sensor chip were activated as described (Amara et al., 1999). The first flow cell was then blocked with 50 μl of 1 M ethanolamine (pH 8.5) and served as a control surface. The three other ones were treated with soluble gp120, gB CMV, or gB HSV (concentration range 1–10 μg/ml in 10 mM acetate buffer [pH 5]). Typically, this procedure permitted the coupling of approximately 250–350 resonance units (RU) of proteins. For binding assays, DC-SIGN CRD was diluted in loading buffer and was allowed to react with the sensor chip (at 30 μl/min). In a typical analysis, DC-SIGN CRD (0.13 to 1 μM, see legend to Figure 7D) was injected over the four flow cells for 8 min, after which the complexes were rinsed with buffer to analyze the dissociation phase. The surface was then regenerated with a 6 min pulse of running buffer containing 50 mM EDTA instead of $CaCl_2$. Sets of sensorgrams were analyzed using the BIAevaluation 3 software.

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DC-SIGN (CD209) Mediates Dengue Virus Infection of Human Dendritic Cells

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Abstract

Dengue virus is a single-stranded, enveloped RNA virus that productively infects human dendritic cells (DCs) primarily at the immature stage of their differentiation. We now find that all four serotypes of dengue use DC-SIGN (CD209), a C-type lectin, to infect dendritic cells. THP-1 cells become susceptible to dengue infection after transfection of DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN), or its homologue L-SIGN, whereas the infection of dendritic cells is blocked by anti–DC-SIGN antibodies and not by antibodies to other molecules on these cells. Viruses produced by dendritic cells are infectious for DC-SIGN– and L-SIGN–bearing THP-1 cells and other permissive cell lines. Therefore, DC-SIGN may be considered as a new target for designing therapies that block dengue infection.

Key words: receptor • flavivirus • lectin • antigen-presenting cells • virus receptor

Introduction

Dengue virus (DV)* is the most common human arbovirus infection worldwide. It is an emerging and volatile public health concern. DV is composed of four antigenically distinct serotypes: DV 1, 2, 3, and 4 (1). All serotypes cause human disease; viremia is detected early, in essentially all DV cases at the onset of symptoms (2). Although most infections are generally mild, a complicated DV infection can result in dengue hemorrhagic fever and dengue shock syndrome. These life-threatening complications usually occur after a second DV infection with a heterologous strain (3). Epidemiologic evidence indicates that the immune-mediated enhancement of infection in the presence of a waning heterologous antibody is the underlying mechanism (4, 5). This process is referred to as antibody-dependent enhancement and was first reported over 30 yr ago (6, 7). The phenomenon of antibody-dependent enhancement notwithstanding, the fact remains that no long-term cross protection is conferred by any dengue serotype (8). This makes the prospects of vaccine design demanding and creates an impetus for new types of therapy.

The design of strategies to counter DV infection requires information on cellular sites and mechanisms of infection. However, limited data are available to establish the major sites of DV replication in vivo. In natural infection, DV is deposited by the mosquito vector into the skin during a blood meal. Recent studies (9–12) indicate that immature dendritic cells (DCs), which are normal residents of the skin, support infection with DV, and that this infection is not altered by a DV-enhancing immune serum (10). Cellular receptors for DV also are not well-defined. Heparan sulfates (13), LPS/CD14-associated binding proteins (14), and

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*Abbreviations used in this paper: DC, dendritic cell; DC-SIGN, DC-specific ICAM-3 grabbing nonintegrin; DV, dengue virus; HIV-1, HIV-type 1; ICAM, intracellular adhesion molecule; L-SIGN, liver/lymph node–specific ICAM-3–grabbing nonintegrin; MCM, monocyte-conditioned media; MFI, mean fluorescence intensity; MOI, multiplicity of infection; NS1, nonstructural protein 1; THP DC-SIGN, DC-SIGN–transfected THP.

other glycoproteins (15, 16) have been proposed as cellular receptors for DV, but these would not explain our prior findings (9) that DCs and not monocytes or macrophages are preferentially infected with DV.

Certain subsets of DCs, especially those susceptible to infection with DV in culture (9–12), express DC-specific intracellular adhesion molecule (ICAM) 3–grabbing nonintegrin (DC-SIGN; reference 17). This C-type lectin allows direct infection of DCs with Ebola virus (18, 19), human cytomegalovirus (20), *Leishmania pifanoi* amastigotes (21), and *Mycobacterium tuberculosis* (22–24). DC-SIGN also accounts, in large part, for the capacity of DCs to capture and retain HIV-type 1 (HIV-1) for the infection of T cells (17, 25–28). Using primary human DCs naturally expressing DC-SIGN, as well as cell lines transfected with DC-SIGN and its homologue liver/lymph node–specific ICAM-3–grabbing nonintegrin (L-SIGN), we show extensive infection with DV as a direct result of expression of these lectins. Because DC-SIGN–mediated DV entry allows for productive infection, releasing infectious virions capable of transmitting DV infection to susceptible cells, these results make DC-SIGN a logical new candidate for interrupting DV infection in humans.

Materials and Methods

Viral Stocks and Infection. All viral stocks and cell lines were mycoplasma-free. The dengue-2 strain, S16803, was grown in an African green monkey Vero cell line (American Type Culture Collection), and cell-free supernatants with a titer of 10^6–10^7 PFU/ml were used as virus stocks. In some experiments, DV 1, 3, and 4 from two sources were also used. The isolates were either nonattenuated (not passaged in primary dog kidney cells) and grown in the lab in C6/36 mosquito cells and Vero cells, or the viruses were low passage clinical isolates from Bandung, Indonesia and grown in C6/36 mosquito cells after isolation. For DV infection, the cells were exposed to DV for 2 h with a multiplicity of infection (MOI) of 0.02–1. The exposed cells were washed with a complete medium (cRPMI consisting of RPMI 1640 [Quality Biologicals] supplemented with 10% heat-inactivated FCS [PAA Laboratories, Inc.], 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin [Quality Biologicals]) at least twice to remove excess virus. For antibody-blocking studies, we tested azide-free anti–human-DC-SIGN1 mAb (clone 120507), anti–DC-SIGN2 (clone 120612), anti–L-SIGN (clone 120604; R&D Systems), anti-CD11a (lymphocyte function–associated antigen 1, LFA-1), anti-CD58 (LFA-3), anti-CD74 (invariant chain), and matched isotype controls (Becton Dickinson). The test cells were pretreated with 2–20 μg/ml of the indicated mAb for 60 min at 37°C before exposure to DV.

Monocyte-derived DCs. PBMCs were cultured as described previously (29) with some modifications. In brief, leukapheresis blood from healthy donors was layered over Ficoll-Hypaque and centrifuged to isolate the mononuclear cells, which were adhered to Petri dishes for 60 min at 37°C. After six to eight washes with complete media, the adherent cells were cultured in 10 ml cRPMI with 800 U/ml rhuGM–CSF (Immunex) and 1,000 U/ml rhuIL-4 (R&D Systems). Cytokines were added every other day. To produce mature DCs, 20% (vol/vol) monocyte-conditioned media (MCM) was added to the cells for an additional 2 d. MCM was prepared as described previously (29). The appropriate phenotype of immature and mature DCs was confirmed by cytofluorometry before each experiment. Specifically, immature and mature DCs lacked CD3, CD14, CD20, and CD56, but expressed high levels of MHC class I, class II, and CD1a; only mature cells expressed high levels of CD25, CD83, and CD86 as described previously (30).

THP-1 Human Monocytic Cell Lines. We were provided with DC-SIGN–transfected THP-1 (THP DC-SIGN), THP DC-SIGN Δ35 (fully truncated cytoplasmic tail), and THP-1 cells by Dr. D. Littman (New York University School of Medicine, New York, NY; reference 27) and L-SIGN/DC-SIGNR expressing THP (THP L-SIGN) by Dr. V. KewalRamani (National Cancer Institute, Frederick, MD; reference 25). All THP-1 cells were grown in complete media.

Monitoring Infection with DV. The DV or mock-infected cells were cytospun onto slides, air-dried, and fixed with 4% paraformaldehyde. Specific anti-DV mAbs, 2H2, or 3H5 (anti-DV envelope complex) or anti-DC-SIGN (120507) were applied after permeabilization with 1% saponin. After several washes, the appropriate secondary antibodies were added; i.e., for immunofluorescence, directly conjugated goat anti–mouse Alexa Fluor 546 or 488 (Molecular Probes) or for immunocytochemistry, biotinylated horse anti–mouse followed by Vectastain ABC alkaline phosphatase or peroxidase kits (Vector Labs). Nuclei were labeled with DAPI (4′, 6′-diamidino-2-phenylindole · 2HCl; Sigma-Aldrich) for immunofluorescence. Matched isotype control antibodies were used in both mock-infected and DV–exposed cells to assess background staining. The slides were mounted and analyzed using either standard light microscopy or a deconvolution microscope (AX-70 laser scanning microscope; Olympus). To quantify the infection virus, a Vero cell plaque assay was performed as described previously (31). Six 10-fold serial dilutions ($1{:}10$–$1{:}10^6$) were made for each supernatant sample and inoculated into duplicate wells of six-well tissue culture plates containing confluent Vero cell monolayers. After virus adsorption for 1 h, the Vero monolayers were overlaid with complete MEM media (Cellgro) containing agarose (Invitrogen) to restrict dissemination of progeny virions. The cells were incubated for 5 d at 37°C in a 5%-CO_2 incubator and overlaid with the vital stain, neutral red (Sigma-Aldrich). Plaques were counted by visual inspection at 24 and 48 h after neutral red overlay to determine the number of plaque-forming units of DV per milliliter of supernatant.

Flow Cytometry. To characterize DCs, a FACS® Calibur (Becton Dickinson) was used to monitor surface staining with a panel of PE-conjugated mAbs to HLA-DR, CD80, CD86, CD3, CD14, CD20, CD25 (Becton Dickinson), CD83 (Immunotech), CD11c, CD1a (BD Biosciences), and matched isotype controls. To detect intracellular DV antigens, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin, stained with FITC-conjugated–2H2 (anti–DV envelope complex mAb) and/or FITC-conjugated–7E11 (anti–DV-NS1 mAb, first nonstructural protein), with antibodies provided by R. Putnak (Walter Reed Army Institute of Research, Washington, DC).

Statistical Analysis. This was performed with StatView 5 (SAS Institute).

Results

Cell-surface DC-SIGN Expression Correlates with DV Infection Rates. We used flow cytometry to follow the expression of DC-SIGN and infection with DV. We suspected that DC-SIGN (CD209) might serve as a DV

receptor. For example, prior work showed that immature DCs were much more susceptible to DV infection than their mature counterparts (5–10-fold higher levels; reference 9), and we found that the expression of DC-SIGN mean fluorescence intensity (MFI) was threefold higher on immature DCs (MFI = 175 ± 49, $n = 4$) compared with mature DCs (MFI = 60 ± 3, $n = 3$; Fig. 1 A) in agreement with other reports (18, 27, 32, 33). To study the role of DC-SIGN in DV entry, we used a DC-SIGN–transfected THP-1 (THP DC-SIGN) cell line, and the corresponding nontransfected THP cells as controls (27). We confirmed high levels of DC-SIGN on the transfected cells and no DC-SIGN expression on the nontransfected cells (Fig. 1 A). When these different cell types were exposed to DV (MOI = 1) and evaluated for infection 48 h later using a DV-specific monoclonal antibody (2H2) that binds to a dengue envelope complex protein, only the DCs and the THP DC-SIGN transfectants were infected (Fig. 1 B). In multiple experiments, the amount of infection of each cell type was directly correlated ($r = 0.89$) with the level of DC-SIGN expression (Fig. 1 C). These data implicate DC-SIGN in DV infection.

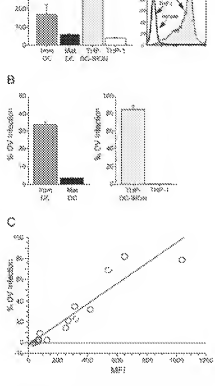

Figure 1. Cytofluorometry of DV infection of DC-SIGN–expressing cells. (A) The MFI of DC-SIGN surface expression on DC and THP-1 cells is shown (left). The bars represent means (± SEM) of at least three independent experiments. The top right histogram shows the relative DC-SIGN expression (MFI) on a representative donor's immature (shaded) and MCM-matured DCs (heavy line). The bottom right histogram shows relative DC-SIGN on the surface of THP DC-SIGN (shaded) and THP-1 cells (heavy line). Matched isotype controls are represented by the light dashed line. (B) The mean (± SEM) percentage of DV infection of immature and mature DC (left) and THP DC-SIGN and THP-1 (right) after intracellular staining for dengue envelope antigen (2H2) in at least three independent experiments. (C) Positive correlation between DC-SIGN expression (MFI) on the x axis and percent DV infection as determined by 2H2 binding on the y axis on both DC and THP cells ($n = 16$).

THP DC-SIGN Can Be Infected with All Four Serotypes of DV. We extended the study of DV infection to two different DV-specific monoclonal antibodies, the previously mentioned 2H2 anti–envelope complex antibody and the 7E11 antibody, to a nonstructural protein 1 (NS1) that is expressed during viral replication but is not a component of the virion. Infection, as assessed by the binding of monoclonal antibodies, was proportional to the dose of added virus (Fig. 2 A). The 2H2 binding typically was slightly higher than the 7E11 binding and again, no infection was detected in the nontransfected THP with either antibody. Kinetic studies with the DEN-2 virus (MOI = 0.2) were used to determine the peak time for detection of infection (Fig. 2 B). The optimal time point was at 48 h after viral exposure, which is consistent with previous studies in primary DCs (9, 11, 12). All studies used this 48-h time point, unless otherwise noted. All four DV serotypes were able to infect THP DC-SIGN transfectants, as previously shown in immature DCs (Fig. 2 C; reference 9). Of note, both low passage clinical isolates (two passages) and more lab-adapted isolates were able to infect at similar levels. When DV infection was evaluated in THP cells transfected with L-SIGN, a close relative of DC-SIGN (25), high levels of infection were again observed with all four serotypes (unpublished data). To inspect DV infection at the cellular level, we looked for DV antigens (2H2; red) using immunofluorescence and confocal microscopy in DC-SIGN–bearing cells (Fig. 2 D, green) at early and late time points, 2 and 24 h, respectively (Fig. 2 D). Newly produced virions accumulated in both DCs and THP transfectants at the later time point (Fig. 2 D, bottom), which is consistent with prior EM observations that replicating DV (at 24–48 h) is found in the endoplasmic reticulum of DCs (9, 12). The presence of the replicating virus within the DC-SIGN–bearing cells was confirmed by staining viral antigens with 2H2, using immunohistochemistry techniques. Abundant viral antigen was noted in the cytoplasm (Fig. 2 E, red) in DC-SIGN–bearing cells (Fig. 2 E, blue) at 48 h (but not at 2 h) for both immature DC and THP DC-SIGN (Fig. 2 E) and THP L-SIGN (unpublished data). No viral antigens were detected in non DC-SIGN–bearing cells (unpublished data). Therefore, transfection of THP cells with DC-SIGN and L-SIGN renders the cells permissive to infection with all DV serotypes.

Anti–DC-SIGN Monoclonal Antibodies Block DV Infection in Dendritic Cells and THP DC-SIGN Cells. To further evaluate DC-SIGN as a potential DV receptor, we used two different anti–DC-SIGN antibodies (clones 120612

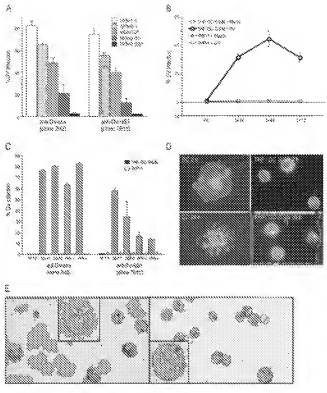

Figure 2. Dose and time dependence of DV infection in THP DC-SIGN cells. (A) Titration of DV2 infection in THP DC-SIGN and THP-1 2 d after infection. The infected cells were stained for DV envelope antigen (clone 2H2) and the NS1 (clone 7E11) and infection was determined by calculating the percentage of fluorescence-positive cells. (B) Kinetics of DV2 infection in THP DC-SIGN and THP-1. The infected cells were harvested at t = 0, 24, 48, and 72 h and stained with 2H2 and 7E11. (C) DV infection rates of all four DV serotypes in THP DC-SIGN and THP-1, using DV 1, 2, 3, and 4. Data are averages of two independent experiments. (D) Representative immunofluorescence experiment at two time points (2 h, top, and 24 h, bottom) showing bound DV envelope complex antigens (red) in DC-SIGN (green)–bearing DC (left) and THP-DC-SIGN (right) cells. Nuclei were labeled with DAPI stain. (E) Representative immunohistochemistry experiment showing bound intracellular DV envelope complex antigens (dark red) in DC-SIGN–bearing cells (blue). The left (immature DC) and right panels (THP DC-SIGN) show cells infected with DV. Original magnifications at 200 (insets, 600).

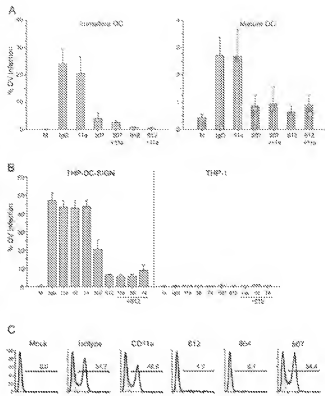

Figure 3. Blocking studies of DV infection in DCs and THP-1. (A) Comparison of percent DV2 infection rate of immature (left) and mature DCs (right) in the presence and absence of anti–DC-SIGN mAbs (clones 120507-specific or 120612–cross-reactive), anti-CD11a, or an irrelevant matched isotype control. (B) Comparison of percent DV2 infection rate of THP DC-SIGN and THP-1 in the presence and absence of specific anti-DC-SIGN mAbs, anti-CD11a, anti-CD58, anti-CD74, or an irrelevant matched isotype control. Data are means (± SEM) of four independent experiments. (C) A representative blocking experiment (one of two) in the THP L-SIGN cells in the presence and absence of specific anti–DC-SIGN or L-SIGN mAbs (120604), anti-CD11a, or an irrelevant matched isotype control.

and 120507). Preliminary antibody titration experiments were performed, which indicated that a range of 2–20 μg/ml antibody was sufficient to reduce infection of the DC (unpublished data). All subsequent blocking experiments were conducted using 2 μg/ml anti–DC-SIGN antibody and matched isotype control antibodies including anti-CD11a. In multiple experiments, both DC-SIGN antibodies independently and significantly blocked DV infection of either immature or mature DCs at 2 μg/ml (Fig. 3 A). We used similar experimental conditions for blocking DV infection of THP cells (Fig. 3 B). We added two other relevant antibodies that react strongly with the cells: anti-CD58 (LFA-3) and anti-CD74 (invariant chain). The levels of DV infection were unaltered in the presence of antibodies to CD58, CD74, or CD11a. Interestingly, we found that the 120507 clone was less effective at blocking DV infection (reduced by 50%) when compared with the "cross-reactive" 120612 clone (reduced by 90%) in the THP DC-SIGN cells. The THP L-SIGN cells were readily blocked using either the cross-reactive clone 120612 (>75% reduction) or the L-SIGN–specific clone 120604 (95% reduction; Fig. 3 C). Thus, DV infection of DCs, DC-SIGN, or L-SIGN–transfected cells can be significantly blocked by antibody-selective targeting of an epitope shared by DC-SIGN and L-SIGN.

The Cytoplasmic Domain of DC-SIGN Enhances but Is Not Essential for Infection. To gain information on the mechanism of DC-SIGN function, we considered a THP DC-SIGN cell line in which the DC-SIGN molecule lacked its cytoplasmic domain (THP DC-SIGN Δ35). The cytosolic tail of DC-SIGN mediates endocytosis and is important for sequestering and transmitting HIV-1 when DCs are exposed to low doses of virus (27). DC-SIGN expression levels on the wild-type and THP DC-SIGN Δ35 cells were comparable (300–500 MFI). We found a significant reduction in viral entry in the THP DC-SIGN Δ35 (75% reduc-

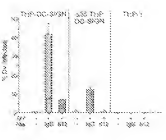

Figure 4. Internalization of DV. Comparison of DV infection of THP DC-SIGN, THP DC-SIGN Δ35 (fully truncated cytoplasmic domain DC-SIGN), and THP-1 in the presence of specific anti–DC-SIGN antibody (clone 120612) or a matched isotype control. Data are the mean (± SEM) of three independent experiments.

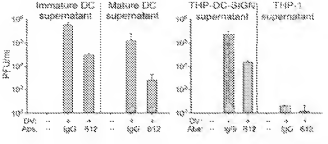

Figure 5. Plaque assays of cell-free culture supernatants show infectivity. Supernatants transmit DV infection from DV-infected DCs (left) and THP DC-SIGN cells (right) to Vero cells. DCs or THPs were pretreated with an anti–DC-SIGN mAb (clone 120612) or a matched isotype control and exposed to DV for 2 h. Virus and mAb were washed away, and the supernatants were collected 48 h later. Plaque assays were used to determine PFUs in the collected supernatants. Data are the averages of two independent experiments.

tion) after DV exposure (Fig. 4). Nevertheless, the residual DV infection could be completely abrogated in the presence of the anti–DC-SIGN antibody (clone 120612). This residual infection might have resulted from the constitutive entry of the truncated DC-SIGN receptor during bulk phase endocytosis, which is active in the cells we studied.

Spreading Infection of DCs Is Inhibited by Anti–DC-SIGN Antibodies. To determine if DV infection of DCs produced infectious virions, we performed a standard DV plaque assay (31) in susceptible Vero cells. We infected DCs or THP DC-SIGN/THP L-SIGN cells for 2 h in the absence or presence of blocking anti–DC-SIGN mAbs, washed, cultured the cells for 48 h to allow for production of infectious virus, and checked the 48-h supernatants for infection of Vero cells. The data showed that supernatants from the DC-SIGN–bearing cells induced plaque formation and transmitted DV infection; furthermore, the "trans" DV infection was significantly reduced (>1 log drop [PFU/ml]) when the initial infection of DCs had been performed in the presence of anti–DC-SIGN antibodies (Fig. 5).

Discussion

This paper identifies DC-SIGN and L-SIGN as mediators of DV infection. DC-SIGN is expressed primarily by a subset of DCs, whereas L-SIGN is expressed by certain endothelial cells, especially sinusoidal endothelium in liver and lymphoid organs and placental capillaries. We suggest that DC-SIGN allows immature DCs to capture and replicate DV after transmission from the mosquito vector. It is not clear if standard targets for DV infection, e.g., monkey Vero cells and C6/36 mosquito cells, express a functionally equivalent molecule to DC-SIGN or if they use an entirely distinct pathway of infection.

Although DC-SIGN binds at least two normal cellular adhesion molecules, ICAM-3–CD50 (32) and ICAM-2–CD102 (34), much of the research on this C-type lectin has involved its interaction with pathogens. DC-SIGN is proving to be a receptor used by DCs to capture, replicate, and/or transmit many pathogens, at least in culture. DC-SIGN is known to play a critical role in tissue culture by enhancing HIV transmission from DC to T cells (17, 27, 35). Recently, the Ebola virus (18, 19), cytomegalovirus (20), *L. pifanoi* amastigotes (21), and *M. tuberculosis* (22–24) were shown to use DC-SIGN to gain entry into DCs. Not only are large numbers of DCs and transfected THP-1 cells infected quickly with DV, but these cells yield a virus that is infectious for other cells. Thus, very low MOIs are sufficient to bring about a productive infection. In HIV-1 infection, direct infection of DCs is actually minimal at a time that DC-SIGN is allowing the DCs to sequester and transmit HIV-1 to T cells. With cytomegalovirus, like DV, the DCs themselves are infected (cis) and enhance infection of a permissive cell type in trans (20); in Ebola, *L. pifanoi*, and *M. tuberculosis*, it remains to be determined if DC-SIGN promotes spreading infection. Therefore, DC-SIGN likely recognizes "pathogen-associated microbial patterns." One of the consequences of the DC-SIGN–pathogen interaction may be to enhance infection or pathogenesis by allowing the pathogen to enter the endocytic system. Once inside the endocytic pathway, the pathogen either replicates or fuses with the vacuole membrane to enter the cytoplasm. The latter is proposed for a pathogen like DV, where an acidic environment is likely to be required for the fusion of the DV envelope glycoproteins with the host cell membrane (36).

We used specific monoclonal Abs to block infection of DCs and THP DC-SIGN/L-SIGN to prove that viral entry depends specifically on these lectins. Control antibodies that reacted strongly with DCs and THPs (CD11a, 58, and 74) did not block infection. The two receptors, DC-SIGN and L-SIGN/DC-SIGNR, exhibit 77% amino acid identity and likely share ligands (25). We used two anti–DC-SIGN clones: DC-SIGN–specific (120507) and another cross-reactive clone (120612) that binds to shared epitopes on DC-SIGN and L-SIGN/DC-SIGNR. The antibodies all blocked infection in primary DCs and THP DC-SIGN, but the latter were most efficiently blocked with the cross-reactive monoclonal antibody. This suggests that a critical epitope for DV infection involves an epitope shared by DC-SIGN and L-SIGN. However, this does not exclude other possibilities such as antibody-induced steric hindrance of a nearby DV epitope or antibody-induced conformational changes at a distant epitope.

In an extension of the work presented here, we performed neutralization assays with sera from DV-infected

patients and monoclonal antibodies to the DV envelope. Both the patients' sera and monoclonal antibody independently blocked infection of immature DC and THP DC-SIGN cells in a dose-dependent manner (unpublished data). It is possible that antibodies to DV have two contrasting functions. The valuable one, in terms of vaccine design, would be to block primary infection at the level of the DV envelope interacting with DC-SIGN receptors on DCs (and possibly L-SIGN on endothelial cells). The other contrasting function would be for an antibody to enhance infection via macrophages, which express Fc receptors but lack DC-SIGN. Prior work showed that antibody-mediated enhancement of DV infection, using select "enhancing" sera obtained in the waning stages of heterotypic DV infection, is not observed in DCs (10). The presence of DC-SIGN may override the need for other binding mechanisms to enhance infection in DCs.

Highly conserved, relevant epitopes of DV involved in cellular infection need to be identified to advance the development of DV vaccines and therapies. Current DV vaccine strategies need to incorporate all four serotypes due to the potential risk of DV hemorrhagic fever and shock syndromes. Recent public health concerns also extend to the potential use of the viruses that cause hemorrhagic fevers as weaponzied biological agents (37, 38). The possibility of limiting productive infection of important target cells at the level of virus entry, which for DV includes the DC-SIGN and L-SIGN receptors, may be an option worthy of consideration for translation from the laboratory to the clinic.

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L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus

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Target cell tropism of enveloped viruses is regulated by interactions between viral and cellular factors during transmission, dissemination, and replication within the host. Binding of viral envelope glycoproteins to specific cell-surface receptors determines susceptibility to viral entry. However, a number of cell-surface molecules bind viral envelope glycoproteins without mediating entry. Instead, they serve as capture receptors that disseminate viral particles to target organs or susceptible cells. We and others recently demonstrated that the C type lectins L-SIGN and DC-SIGN capture hepatitis C virus (HCV) by specific binding to envelope glycoprotein E2. In this study, we use an entry assay to demonstrate that HCV pseudoviruses captured by L-SIGN+ or DC-SIGN+ cells efficiently transinfect adjacent human liver cells. Virus capture and transinfection require internalization of the SIGN–HCV pseudovirus complex. *In vivo*, L-SIGN is largely expressed on endothelial cells in liver sinusoids, whereas DC-SIGN is expressed on dendritic cells. Capture of circulating HCV particles by these SIGN+ cells may facilitate virus infection of proximal hepatocytes and lymphocyte subpopulations and may be essential for the establishment of persistent infection.

Hepatitis C virus (HCV) is the etiologic agent of non-A non-B hepatitis in humans (1, 2). Only ≈15% of infected individuals clear the virus, and ≈170 million people worldwide are persistently infected with HCV (3, 4). These individuals may remain asymptomatic or may develop chronic hepatitis or cirrhosis, the latter often leading to hepatocellular carcinoma (5). Hepatocytes are the primary target cells for HCV infection (6–8). Virus-like particles have been visualized in liver biopsies of HCV+ individuals (9–11), and *in vitro* infection, albeit inefficient, of primary hepatocytes and hepatoma cells has been documented (12–14). The existence of extrahepatic reservoirs of HCV is suggested by the detection of viral RNA in serum and peripheral blood mononuclear cells of HCV+ individuals (15–17). Both B and T lymphocytes appear to be infected *in vivo*, which is supported by *in vitro* infection of B and T cell lines (7, 8, 18). One study, however, shows that replicating forms of HCV RNA are restricted to hepatocytes, whereas only nonreplicating forms are present in B lymphocytes, and none are in T lymphocytes (6).

HCV envelope glycoproteins E1 and E2 mediate entry into target cells. We and others recently demonstrated that unmodified E1E2 heterodimers reach the cell surface and are incorporated into retroviral pseudoparticles, which can infect primary hepatocytes and some hepatoma cell lines (19–22). Use of the soluble E2 ectodomain as a surrogate model for studying HCV interactions with cell-surface molecules has identified several potential HCV entry receptors, including CD81, scavenger receptor class B type 1, low-density lipoprotein receptor, and glycosaminoglycans (22–24). Several groups, including ours, have shown that CD81 is necessary but not sufficient for HCV pseudovirus entry into target cells (19, 25, 26). Furthermore, we recently demonstrated that CD81 functions as a postattachment entry coreceptor (26). Cellular factors that act in concert with CD81 to mediate HCV binding and entry remain to be identified.

Engagement of specific receptors is required for viral fusion and entry, but adsorption of viral particles to the cell surface can occur through envelope glycoprotein interactions with other molecules (27–33). The C type lectins, DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin; CD209) and L-SIGN (DC-SIGNR; liver and lymph node-specific; CD209L), function as capture receptors for several viruses, including HIV type 1 (HIV-1) (34), Ebola virus (35), cytomegalovirus (36), and dengue virus (37). Both L-SIGN and DC-SIGN have an extracellular C-terminal region that contains a calcium-dependent carbohydrate recognition domain (CRD) and a membrane-proximal heptad-repeat region important for oligomerization (38–41). Capture of viral particles is mediated by the CRD and promotes infection of target cells both in cis and in trans (34, 35, 42, 43). DC-SIGN also recognizes intercellular adhesion molecules 2 and 3, which function as cell-adhesion receptors that regulate transendothelial migration of dendritic cells (DC) from blood to tissues as well as DC–T cell interactions.

We and others have recently demonstrated that recombinant soluble E2, patient-derived HCV virions, and retroviruses pseudotyped with HCV envelope glycoproteins specifically bind to L-SIGN and DC-SIGN (44–46). HCV capture by SIGN molecules depends on the presence of the CRD, indicating that recognition of high mannose oligosaccharides in the viral envelope glycoproteins is critical for binding. The specificity of this interaction is underscored by observations that (*i*) other C type lectins, such as langerin, CD23, and CLEC-1/2, do not bind HCV E2 (45, 46); (*ii*) glycosylated envelope proteins of several viruses show little or no avidity for SIGN molecules (36, 47); and (*iii*) anti-L-SIGN and anti-DC-SIGN mAbs as well as mannan inhibit soluble E2 and HCV capture.

In this study, we used an HCV entry assay to demonstrate that capture of viral particles by L-SIGN+ and DC-SIGN+ cells promotes transinfection of human liver cells. Transinfection is specifically blocked by mannan and antibodies recognizing CRD of SIGN receptors. Similarly, we show that primary human DC mediate transinfection of target cells by a DC-SIGN-dependent mechanism that requires internalization of the receptor–pseudovirus complex. Our results suggest that HCV capture by SIGN molecules plays an important role in viral dissemination to host target organs. In particular, L-SIGN+ liver sinusoidal epithelial cells (LSEC) may facilitate infection of hepatocytes, whereas DC-SIGN+ DC may transmit HCV to hepatocytes as well as subpopulations of B and/or T lymphocytes.

Materials and Methods

Plasmids, Antibodies, and Inhibitors. Construct pcDNA3.1-ΔC-*E1-E2* was used to express HCV envelope glycoproteins. Se-

Abbreviations: HCV, hepatitis C virus; HIV-1, HIV type 1; CRD, carbohydrate recognition domain; DC, dendritic cells; LSEC, liver sinusoidal epithelial cells.

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quences encoding the full-length E1 and E2 (amino acids 132–746), starting with the last 60 amino acids of the capsid (ΔC), were PCR amplified from p90/HCV FL-long pU comprising the genome of infectious HCV isolate H77 (48) and subcloned into pcDNA3.1 (Invitrogen). Putative splice acceptor sites were modified by conservative mutagenesis, as described (21).

mAbs 507D, 604L, and 612X recognizing the CRD of DC-SIGN, L-SIGN, or both lectins, respectively, were purchased from R & D Systems. Anti-HCV E2 mAb 091b-5 was purchased from Austral Biological. Anti-CD81 mAb JS-81 was obtained from Pharmingen. Chloroquine and mannan were purchased from Sigma.

Cell Lines. Unless otherwise specified, cells were purchased from the American Type Culture Collection and grown in DMEM supplemented with 10% fetal bovine serum/1% penicillin/streptomycin/2 mM glutamine. HeLa cells stably expressing L-SIGN or DC-SIGN were generated as described (44) and maintained in DMEM supplemented with 600 μg/ml G418 (Life Technologies, Grand Island, NY). Primary human immature DC were differentiated from peripheral blood mononuclear cells with 1,000 units/ml granulocyte-macrophage colony-stimulating factor (R & D Systems) and 1,000 units/ml IL-4 (R & D Systems) in culture medium for 5 days, as described (49).

Viral Production and Transinfection. Standard calcium phosphate precipitation was used to transfect 293T cells (1.5×10^6) with NL*luc*+*env*-reporter vector (50) and pcDNA3.1-Δ*C-E1-E2* in a 1:3 ratio. NL*luc*+*env*- encodes an $HIV\text{-}1_{NL4.3}$ envelope-deficient genome expressing *luc* instead of *nef*. Cell culture supernatants, containing HIV-1 particles pseudotyped with HCV E1E2 envelope glycoproteins, were collected 48 h posttransfection and cleared of cellular debris by low-speed centrifugation.

Parental HeLa cells or L-SIGN+ or DC-SIGN+ transfectants (2×10^4) were incubated with 200 μl of viral supernatant for 2 h at 37°C. Alternatively, primary DC (10^5) were incubated under similar conditions, at 37°C or at 4°C, with 1 ml of viral supernatant. For inhibition experiments, cells were incubated with mAbs (10 μg/ml), sera from HCV+ or HCV− individuals (1:100), chloroquine (50 μM), or mannan (20 μg/ml) for 30 min at 37°C before addition of viral supernatants. Alternatively, anti-CD81 mAb JS-81 was added to cells after virus capture by DC. After washing three times with serum-free medium to remove unbound virus, cells were cocultured with Huh-7 target cells (4×10^4) for an additional 48 h at 37°C. Luciferase activity [relative light units (R.L.U.)] was measured in cell lysates by using the Luciferase Assay System (Promega) according to the manufacturer's instructions.

Virus-Binding Assay. Binding of HCV pseudoviruses to HeLa cells expressing L-SIGN or DC-SIGN or to DC was performed as described by Gardner *et al.* (44). Briefly, adherent target cells (10^4) were washed in buffer (20 mM Tris-HCL, pH 8.0/150 mM NaCl/1 mM $CaCl_2$/2 mM $MgCl_2$/0.5% BSA) and incubated with an equal volume of viral supernatant for 1 h at 37°C with gentle agitation every 15 min. Alternatively, cells were incubated with anti-L/DC-SIGN mAb 612X (10 μg/ml) or mannan (20 μg/ml) for 30 min at room temperature before addition of viral supernatants. After washing five times with adherence buffer to remove unbound virus, cells were lysed according to the manufacturer's instructions for the preparation of viral RNA (Qiagen, Valencia, CA). Cell lysates were analyzed for HIV RNA content with the HIV Ultrasensitive Amplicor Assay (LabCorp, Burlington, NC) or p24 content by using the Coulter HIV-1 p24 antigen assay (Beckman Coulter).

E2-Binding Assay. Binding of soluble E2 was performed as described (44). Briefly, primary DC (5×10^5) were preincubated with mannan (20 μg/ml) or mAbs (10 μg/ml) for 10 min at room temperature. E2-coated fluorescent beads were prepared with the anti-E2 091b-5 capture mAb and added to cells (20 beads per cell) for 30 min at 37°C. Binding was quantified by flow cytometry.

Table 1. HCV pseudovirus binding to SIGN+ HeLa cells

	L-SIGN		DC-SIGN	
	p24, pg/ml	Inhibition, %	p24, pg/ml	Inhibition, %
IgG control	121	0 ± 5	160	0 ± 4
mAb 612X	43	59 ± 8	109	30 ± 11
Mannan	23	84 ± 13	49	73 ± 6

L-SIGN+, DC-SIGN+, or parental HeLa cells were incubated with a nonspecific murine IgG (10 μg/ml), anti-L/DC-SIGN mAb 612X (10 μg/ml), or mannan (20 μg/ml), followed by incubation with HCV pseudovirus-containing supernatants. Cell-associated p24 (pg/ml) was measured by using a Coulter HIV-1 p24 antigen assay and corrected for background binding to parental HeLa cells. Percentages of binding inhibition were calculated and adjusted for background by using the formula 100·[(p24 with inhibitors − p24 HeLa with IgG)/(p24 without inhibitor − p24 HeLa with IgG) × 100]%. Negative values are represented as 0 for ease of interpretation. One representative experiment of three independent experiments is shown. The percentages of binding inhibition are means of three independent experiments ± SD.

Results

Binding of HCV Pseudoviruses to DC-SIGN+ and L-SIGN+ Cells. HeLa cells were modified to stably express similar levels of cell-surface L-SIGN or DC-SIGN and were shown to specifically capture soluble E2 envelope glycoprotein and HCV virions from sera of infected individuals (44). In this study, we first confirmed the ability of L-SIGN+ and DC-SIGN+ HeLa cells to capture HIV-1 particles pseudotyped with HCV envelope glycoproteins E1E2 (referred to as HCV pseudoviruses from here on in the text). Cells were incubated with pseudovirus-containing supernatants, washed extensively, and the amount of cell-associated particles was determined by quantification of HIV-1 Gag protein (p24) in cell lysates. Significantly higher p24 values were found in lysates of L-SIGN+ and DC-SIGN+ cells, compared with parental HeLa (Table 1 and data not shown). Preincubation of cells with an anti-L/DC-SIGN mAb (10 μg/ml) recognizing CRDs of both lectins decreased L-SIGN+ and DC-SIGN+ HeLa cell-associated p24 by 59% and 30%, respectively, compared with control mouse IgG (10 μg/ml) (Table 1). Similarly, mannan (20 μg/ml), which specifically interacts with lectin CRDs, decreased cell-associated p24 by 84% and 73% (Table 1). Comparable results were obtained when HCV pseudovirus binding to HeLa cells and SIGN+ derivatives was quantified with the Amplicor HIV-1 Monitor test (Roche), which detects HIV-1 genomic RNA (data not shown). Taken together, these results indicate that L-SIGN and DC-SIGN molecules capture HCV pseudoviruses through specific interactions between envelope glycoprotein-associated carbohydrates and lectin CRDs.

SIGN-Mediated Transinfection of Huh-7 Hepatoma Cells with HCV Pseudoviruses. HCV pseudoviruses comprise an HIV-1 core and genome, which is envelope glycoprotein-deficient and expresses the *luc* reporter gene. HCV E1E2-mediated entry of these viral particles into primary human hepatocytes and certain hepatoma cells is inhibited by sera from HCV+ individuals as well as an anti-CD81 mAb and certain anti-E2 mAbs (19, 20, 26). This system therefore authentically reproduces the early steps of HCV replication, and we used it to investigate the ability of HeLa cells expressing L-SIGN or DC-SIGN to transfer captured HCV pseudoviruses to entry-permissive human liver (hepatoma) cells. HeLa cells as well as SIGN+ transfectants are resistant to E1E2-mediated entry, because infection with HCV pseudoviruses resulted only in background levels of luciferase

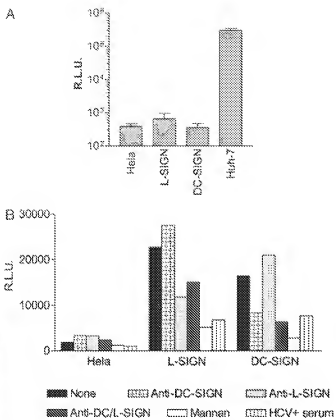

Fig. 1. L-SIGN- and DC-SIGN-mediated transinfection of HCV pseudoviruses into Huh-7 cells. L-SIGN+, DC-SIGN+, parental HeLa cells, or Huh-7 cells were infected with HCV pseudoviruses. Luciferase activities [relative light units (R.L.U.)] were measured in cell lysates 48 h postinfection. Values are means of three independent experiments ± SD. (*A*) L-SIGN+, DC-SIGN+, or parental HeLa cells were incubated with HCV pseudovirus-containing supernatants, washed, and cocultured with Huh-7 cells. Luciferase activity in cell lysates was measured 48 h postinfection. (*B*) Alternatively, DC-SIGN+, L-SIGN+, or parental HeLa cells were preincubated with anti-DC-SIGN mAb 507D (crosshatched bars), anti-L-SIGN mAb 604L (dotted bars), anti-DC/L-SIGN mAb 612X (gray bars), mannan (white bars), HCV+ sera (striped bars), or HCV− sera (data not shown), before addition of pseudoviral supernatants. One representative experiment of three independent experiments is shown.

activity (Fig. 1*A*). Huh-7 hepatoma cells, however, are highly permissive to HCV pseudovirus entry, with luciferase activities typically in the 10^5–10^6 relative light unit (R.L.U.) range (Fig. 1*A*). Preincubation of L-SIGN+ or DC-SIGN+ HeLa with HCV pseudovirus supernatants, followed by removal of unbound particles and coculture with Huh-7 hepatoma cells, resulted in significant transinfection of these cells. Luciferase activities were typically in the 10^4–10^5 R.L.U. range, which corresponds to ≈10% of entry levels obtained by direct infection (Fig. 1*B*). Transinfection mediated by L-SIGN was consistently more efficient than transinfection mediated by DC-SIGN. Coculture of HeLa cells with Huh-7 cells did not result in transinfection (Fig. 1*B*), further demonstrating that viral particles must be captured by SIGN molecules to remain infectious and to be transmitted to target cells.

Transinfection was also measured in the presence of specific inhibitors. Mannan (20 μg/ml) blocked HCV pseudovirus transmission to Huh-7 cells by L-SIGN+ and DC-SIGN+ HeLa by 75% and 85%, respectively (Fig. 1*B*). Pseudoviral entry was also significantly reduced by the anti-L/DC-SIGN mAb 612X (10 μg/ml), with a greater effect on DC-SIGN-mediated transinfection (Fig. 1*B*). As expected, anti-L-SIGN mAb 604L (10 μg/ml) and anti-DC-SIGN mAb 507D (10 μg/ml) inhibited only transinfection mediated by L-SIGN and DC-SIGN, respectively (Fig. 1*B*). Generally, transinfection was inhibited by ≈30–60% with the different mAbs, all of which recognize the CRDs of the lectin receptors. We also ascertained that transinfection depended on HCV envelope glycoproteins by showing that it is inhibited by ≈80% with sera from HCV+, but not HCV−, donors (1:100) (Fig. 1*B* and data not shown). Taken together, these findings show that HCV particles captured by L-SIGN+ or DC-SIGN+ cells via interactions with E1E2 remain infectious and are efficiently transmitted to target cells.

Table 2. Soluble E2 and HCV pseudovirus binding to primary dendritic cells

	Fluorescent cells, %	Inhibition, %	Units/ml	Inhibition, %
IgG control	55	—	65,000	—
612X mAb	13	71 ± 15	17,000	74 ± 8
Mannan	22	66 ± 12	32,000	49 ± 12

Primary DC isolated from an HCV− donor were incubated with a control murine IgG (10 μg/ml), anti-L/DC-SIGN mAb 612X (10 μg/ml), or mannan (20 μg/ml), followed by incubation with fluorescent beads coupled with soluble E2. Binding was measured by flow cytometry and expressed as a percentage of fluorescently labeled cells. Alternatively, DC were incubated with HCV pseudovirus-containing supernatants, and the number of cell-associated HIV-1 RNA copies was quantified by real-time PCR. Percentages of inhibition were calculated as described for Table 1. One representative experiment of three independent experiments is shown. The percentages of binding inhibition are means of three independent experiments ± SD.

Human DC Bind to HCV via E2 and Mediate Transinfection of Liver Cells. We next tested whether DC-SIGN+ primary human DC could specifically capture soluble E2 envelope glycoprotein and HCV pseudoparticles. Peripheral blood mononuclear cells were isolated from an HCV− donor, and monocytes were differentiated into immature DC by treatment with granulocyte–macrophage colony-stimulating factor and IL-4. Soluble E2 was coated onto fluorescent beads conjugated to anti-E2 mAb 091b-5, and binding to DC was analyzed by flow cytometry, as described (44). Over 50% of cells bound E2-coated beads, and binding was inhibited with anti-L/DC-SIGN mAb 612X (10 μg/ml) and mannan (20 μg/ml) by 71% and 66%, respectively (Table 2). In addition, binding of HCV pseudoparticles to DC was detected by RT-PCR with the Ultrasensitive Amplicor Assay. Similar to inhibition of soluble E2 binding, HCV pseudovirus attachment to DC was inhibited with anti-L/DC-SIGN mAb 612X and mannan by 74% and 49%, respectively (Table 2). These results demonstrate that both soluble E2 and HCV pseudoviruses are captured onto primary DC by specifically interacting with DC-SIGN. Variations in potency of inhibition in Tables 1 and 2 could be due to differences in cellular backgrounds, levels of DC-SIGN expression, and use of different assays to detect HCV pseudoparticle binding to cells.

Transinfection of target cells by DC-captured HCV pseudoviruses was also evaluated by using DC derived from two HCV− donors. DC from donor A expressed twice as much DC-SIGN compared with cells derived from donor B, as determined by flow cytometry analyses after labeling with mAb 612X (Fig. 2*A Upper*). DC from both donors were incubated with HCV pseudoviruses followed by washing and coculture with Huh-7 cells. We note that only background luciferase activities were observed in the absence of target cells, confirming that DC are not susceptible to E1E2-mediated pseudovirus entry (Fig. 2*A*). DC derived from donor A were ≈30% more efficient at mediating transinfection of Huh-7 cells than DC derived from donor B (Fig. 2*A*), consistent with higher levels of DC-SIGN expres-

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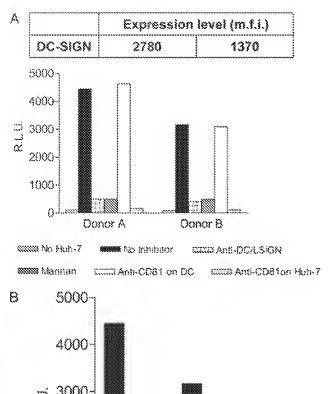

Fig. 2. DC-mediated transinfection of HCV pseudoviruses into Huh-7 cells. Immature human DC were differentiated from peripheral blood mononuclear cells of two HCV − donors (*A* and *B*) by using granulocyte–macrophage colony-stimulating factor and IL-4. (*A*) Expression of DC-SIGN was quantified by flow cytometry and represented as mean fluorescent intensity (m.f.i.) of an anti-DC-SIGN mAb. DC were incubated with HCV pseudovirus-containing supernatants, washed, and cocultured with Huh-7 cells. Luciferase activity was measured in cell lysates 48 h postinfection. Direct infection of DC by HCV pseudoviruses was measured in the absence of Huh-7 cells (dotted bars). DC were also preincubated with HCV pseudoviruses alone (black bars) or in the presence of anti-DC/L-SIGN 612X (crosshatched bars), mannan (gray bars), or anti-CD81 mAb JS-81 (white bars). Alternatively, JS-81 was added to DC and Huh-7 cells after pseudovirus capture (dotted bars). (*B*) Alternatively, DC were incubated with HCV pseudovirus-containing supernatants at 37°C (black bars), 4°C (gray bars), or in the presence of chloroquine (white bars), washed, and cocultured with Huh-7 cells. Luciferase activity was measured in cell lysates 48 h postinfection.

sion. Moreover, transinfection was inhibited by both mannan (20 μg/ml) and the anti-DC/L-SIGN mAb 612X (10 μg/ml), confirming that it occurs through a DC-SIGN-dependent mechanism (Fig. 2*A*). When DC were incubated with the anti-CD81 mAb JS-81 (10 μg/ml) before HCV pseudovirus capture, transinfection of Huh-7 cells was unaffected. However, addition of the anti-CD81 mAb JS-81 (10 μg/ml) to mixtures of DC and target cells after HCV pseudoparticle capture by the former completely inhibited transinfection (Fig. 2*B*). Therefore, CD81 does not play a role in SIGN-mediated virus capture but is still necessary for transinfection of target cells.

Immature DC efficiently internalize and process viruses for presentation to the immune system. DC-SIGN is believed to play a role in virus escape from this process. Consequently, we also investigated the role of virus internalization in DC-mediated transinfection of HCV to target cells. DC were incubated with HCV pseudoviruses at 4°C, which is permissive for attachment but not internalization of virus–receptor complexes. Alternatively, DC-SIGN-mediated capture of HCV pseudoviruses was carried out in the presence of chloroquine, which is a weak base that prevents acidification of endosomes and blocks receptor recycling from the endocytic pathway. DC were then washed and cocultured with Huh-7 cells at 37°C and entry was quantified 48 h later. Incubation at 4°C or in the presence of chloroquine during HCV pseudovirus captures strongly inhibited (>80%) transinfection mediated by DC from both donors (Fig. 2*B*). These results indicate that endocytosis of the DC-SIGN–virus complex plays a critical role in transinfection.

Discussion

The HCV E2 envelope glycoprotein has 11 *N*-linked glycosylation sites, and the majority of oligosaccharides on E2 are high-mannose structures. C type lectin CRDs specifically bind high-mannose *N*-linked oligosaccharides associated with surface components of viruses and bacteria, which are then internalized and routed to lysosomes and MHC class II positive endosomes. Certain viruses, however, are able to escape targeting to lysosomes that occurs for other pathogens (42). Instead, captured viral particles are recycled from nonlysosomal intracellular compartments and are transferred to target cells, where virus-cell fusion is mediated by envelope glycoprotein interactions with bona fide entry receptors. The best-characterized example is HIV-1, which is captured by DC-SIGN+ DC at sites of mucosal exposure. DC-SIGN-mediated internalization of HIV-1 particles into nonlysosomal compartments appears to protect the virus from degradation during transport to lymphoid organs, where it is transmitted to $CD4^+$ $CCR5^+$ lymphocytes (34, 42, 43). Other viruses, such as Ebola, dengue, and Sindbis, may use similar mechanisms to infect target cells either in cis or in trans (35, 37, 51).

HCV replicates in hepatocytes, and several studies have documented HCV replication in B and T lymphocytes (9, 10, 52–55), but the determinants of HCV tropism are unknown. We recently demonstrated that CD81 serves as an obligate entry coreceptor for HCV (26). However, CD81 is ubiquitously expressed on human cells and cannot account for the restricted tropism of HCV (56). Instead, other HCV receptors may be expressed on hepatocytes and some lymphocytes, thereby restricting viral entry into these cell types. We and others have shown (44–46) that HCV envelope glycoprotein E2 as well as HCV virions and pseudoviruses specifically bind to L-SIGN+ and DC-SIGN+ cells. In this study, we demonstrate that expression of SIGN receptors does not render cells permissive to infection by HCV pseudoviruses but does enable them to mediate infection of human liver cells in trans. The specificity of this process is underscored by inhibition of pseudovirus capture and transinfection with mannan and mAbs against CRDs of L-SIGN and DC-SIGN. Moreover, primary human DC also mediate DC-SIGN-dependent transinfection of liver cells, which requires internalization of the DC-SIGN–pseudovirus complex. An additional determinant of HCV tropism therefore may be L-SIGN- and DC-SIGN-mediated targeting of the virus to the liver and lymph nodes.

L-SIGN is expressed mainly by endothelial cells in liver and lymph node sinusoids, whereas DC-SIGN is expressed mostly on myeloid-lineage DC (38, 39, 57, 58). The endothelium of liver sinusoids (specialized capillaries) serves as an active barrier between circulating blood and hepatocytes. LSEC lack a basal lamina and comprise unique pores (fenestrae), which act as filters for fluids, solutes, and particles that are exchanged between the sinusoidal lumen and hepatocytes (59). Another functional characteristic of LSEC is their effective uptake of a wide variety of substances from the blood by receptor-mediated endocytosis. Moreover, some of these substances are actively transferred across the endothelial barrier to surrounding tissues by transcytosis. We postulate that L-SIGN-mediated capture of circulating HCV particles by LSEC results in transcytosis of the virus across the endothelial barrier, thereby concentrating infectious particles and placing them in direct contact with entry-permissive hepatocytes. Similarly, DC-SIGN+ DC, which are present in mucosal compartments and circulating blood, may capture HCV at these sites of primary infection. DC are antigen-presenting cells and interact directly with both B and T lymphocytes (60–63), some of which may be susceptible to HCV infection. Also, DC have been shown to migrate from the blood to the liver by translocation across the endothelium of hepatic sinusoids and may thus transport HCV to hepatocytes (64).

The precise mechanism of SIGN-mediated transinfection is unknown. We show that, similar to HIV-1, HCV transinfection mediated by DC depends on internalization of viral particles. Treatment of DC with chloroquine results in inhibition of transinfection, indicating that pH changes within intracellular compartments are critical for the process. This finding is highlighted by the observation that HCV pseudovirions that have been captured but not internalized (by incubation at 4°C) are not transmitted to target cells. It is possible that acidification of the SIGN–virus complex leads to its dissociation, thereby facilitating transfer of infectious particles to receptor-expressing target cells. Even though HCV pseudoviruses enter cells by low pH-dependent receptor-mediated endocytosis (20), it is remarkable that SIGN-mediated internalization does not prematurely expose cryptic E1E2 fusion domains and inactivate the virus. This may indicate that HCV envelope glycoproteins are insensitive to low pH-induced modifications in the absence of receptor-induced conformational changes. In addition, DC can selectively retain antigens in their native form inside neutral to mildly acidic vesicles (65, 66).

Conclusion

HCV envelope glycoproteins participate in a complex cascade of interactions with specific cell-surface molecules to target and enter host cells. Binding of E1E2 to the CD81 coreceptor and other molecules on hepatocytes results in viral fusion and entry. In addition, HCV envelope glycoproteins bind to at least two C type lectins, L-SIGN and DC-SIGN, expressed on LSEC and DC, respectively, which interact with HCV target cells. SIGN-mediated transinfection of hepatocytes with HCV, which are not in direct contact with circulating blood, may be essential for establishment of persistent infection.

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